



TITLE:

Identification of the bioactive peptides in the rat brain with the hydra bioassay system and analysis of their functions(Dissertation_全文)

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CITATION:

Manabe, Yasuko. Identification of the bioactive peptides in the rat brain with the hydra bioassay system and analysis of their functions. 京都大学, 2001, 博士(農学)

ISSUE DATE:

2001-03-23

URL:

<https://doi.org/10.11501/3183489>

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Yasuko Manabe

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ABBREVIATIONS

TBF	tentacle ball formation
GSM	S-methylglutathione
PIPES	piperazine-N,N'-bis-(2-ethanesulfonic acid) sodium salt.
TGF-β	transforming growth factor beta
CSF	cerebrospinal fluid
CRF	corticotropin-releasing factor
✓ a CSF	artificial cerebrospinal fluid
DBI	diazepam binding inhibitor
CMC saline	saline containing 0.5 % carboxymethyl cellulose
i.p.	intraperitoneally
i.c.v.	intracerebroventricularly
FG7142	N-Methyl- β -carboline-3-carboximide



GENERAL INTRODUCTION

Central nervous system is the most important system in the vertebrates. Information processing system in the brain is so complicated that it has not been known well yet. We need much multidirectional research to elucidate that system. We focused on neurotransmitter, which play an important role in the information processing system in the brain. The releasing rate of neurotransmitter varies with the external stimulation, and the concentration of neurotransmitter in cerebrospinal fluid (CSF) reflect the change of physiological condition. Thus, we tried to elucidate the variation of neurotransmitter in CSF in connection with the physiological change. However, the stumbling block has been a lack of studying method. There is no way to screen a minute amount of bioactive substance in CSF.

Hydra bioassay system makes it possible to detect peptide-like substances (Hanai, 1981). This assay utilizes a tentacle ball formation (TBF), a component of the feeding response of hydra, elicited by S-methylglutathione (GSM). TBF elicited by GSM was modulated by a number of biologically active peptides in a specific way to individual peptides (Fig. 1), and is useful in investigating biologically active peptides in a complex biological sample (Hanai et al., 1987; Hanai et al., 1989; Manabe et al., 2000). In this assay system, peptides and other proteinaceous substances primarily produce the most profound modulation, while catecholamine and other low molecular weight neurotransmitter substances have no effect. We applied the hydra bioassay system to explore a possible biologically active substance in connection with the physiological change.

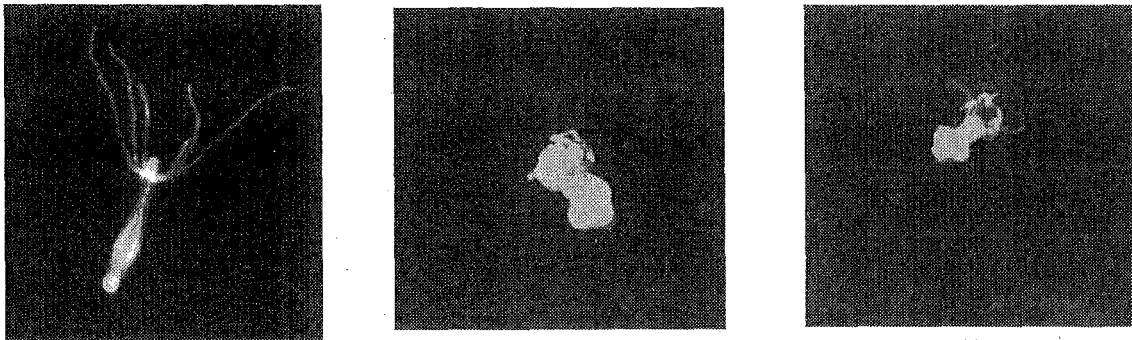


Fig. 1 TBF of Hydra. (left) Resting Hydra (not stimulated by GSM). (middle) Hydra showed TBF by GSM. (right) The suppression of the TBF by the biologically active peptide.

At beginning of this study, we tried to construct Hydra bioassay system to deal with CSF. Hanai et al. has already constructed the assay system (Hanai, 1981; Hanai, 1990; Hanai, 1995; Hanai et al., 1998). However, the observation of TBF is sensitive to assay condition such as temperature, water, and quality of feed. Careful and prompt observation of TBF is required to discriminate TBF despite of any assay condition. Thus, we constructed the best

condition for assay.

Second, we applied this assay to find a possible biologically active substance relevant to fatigue. Perception of fatigue occur within the brain, these fatigue named central fatigue (Bailey et al., 1992; Bailey et al., 1993; Blomstrand et al., 1988; Blomstrand et al., 1989; Davis et al., 1997; Jakeman, 1998; Newsholme et al., 1991). It was proposed in general that the cause of this fatigue was related to the increase of serotonin level in the brain. However, fatigue was integral phenomenon, hence we assumed that the perception of that was not only the change of neurotransmitters (especially serotonin) but also participation of the some factors. We have reported that the injection of high molecular weight fraction of CSF from rats after the exhaustive physical exercise into the brains of sedentary mice decreased the spontaneous motor activity, indicating that a high molecular weight component also may be responsible for the decrease in the motor activity of mice (Inoue et al., 1998). Thus, we tried to identify the responsible biologically active component by using the hydra bioassay to test biologically active substances.

Third is a study about relationship between a bitter taste and a substance in the brain. At present, dopamine (Mark et al., 1994; Martel et al., 1996; Schneider, 1989), opioids (Doyle et al., 1993; Levine et al., 1985; Rideout et al., 1996) and benzodiazepines (Berridge et al., 1995; Cooper, 1982; Gray et al., 1995) affect the palatability of foods, while studies of substances released in the brain after stimulation by an aversive taste are quite limited. Thus, we searched for a substance released in the brain from CSF of rats after they had experienced a quinine-HCl solution using hydra bioassay.

This study was undertaken to identify the released peptide in CSF that was related to physiological change using the hydra bioassay. (1) What is the released substance related to fatigue? and (2) Can the sensation of aversive taste explain the substance in the brain?

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CHAPTER I Construction of screening system for bioactive peptides with the hydra bioassay

Introduction

S-methylglutathione (GSM), a stimulant as potent as the reduced glutathione, elicited tentacle ball formation (TBF), a component of the feeding behavior of Hydra, which was modified in the presence of various peptides. Of all the components of the Hydra feeding behaviors, only TBF was modified in the presence of various peptides. Tentacle ball formation has an interesting feature to be sensitively modulated by various biologically active peptides including platelet-derived growth factor (Hanai et al., 1987), acidic fibroblast growth factor (aFGF) (Hanai et al., 1989). The modulation (mainly suppression) was observed at different stimulatory concentrations of GSM. These previous observations were obtained with Hydra under different culturing conditions from the present. At present the trypsin treatment of live Hydra is required to observe the TBF (Hanai and Matsuoka, 1995). It is likely that a subtle change in culturing conditions of Hydra affects behaviors elicited by GSM since GSM induces so many different, but mutually related behavioral components in Hydra.

In this study, we report the culture conditions of Hydra, under which TBF occurs efficiently as the TBF, and at the same time does the modulation of the response by aFGF and other synthetic peptides. Further, we describe a novel effect of TGF- β ; it eliminated the suppression of the TBF caused by other biologically active substances, while almost all substances examined suppressed the TBF. We also examined the effects of TGF- β and closely related peptides on the TBF in detail.

Materials and Methods

Hydra culture

Hydra japonica was cultured as described before (Hanai, 1998). It was fed every two days with *Artemia naupli* (Argentemia Gold, Argent, Redmond, WA), which was hatched in solution of 30 g/L common salt (>99% NaCl, Japan Tobacco, Tokyo, Japan) containing 1 ng/L ZnCl₂ and 0.3 g/L LiCl (except for the experiment examining salt compositions, see below) by aeration for 1 day at 28-30 °C. A portion of Hydra were transferred to a new bat from the mass culture bats for behavioral assay, and cultured for another 4 days before the behavioral test. These Hydra were fed on the first and the third day with *Artemia naupli* which were hatched in a solution of 30 g/L common salt supplemented with 4.0 g/L of MgCl₂•6H₂O by aeration for 1 day at 28-30 °C. Hydra, which was fed with *Artemia* hatched in the media supplemented with MgCl₂, showed the TBF that was more easily modulated in the presence of peptides.

Effect of salt composition of Artemia hatching solution

Artemia were hatched in the solution containing 30 g/L common salt (from Japan Tobacco mentioned above), 0.3 g/L LiCl, and one of following metal salts 10 ng/L: NaVO₃, MnCl₂•4H₂O, FeCl₃•6H₂O, CoCl₂•6H₂O, NiCl₂•6H₂O, CuSO₄•5H₂O, ZnCl₂, NaSeO₃, and Na₂MoO₄. The effects of the varied concentrations of ZnCl₂ were examined in the presence and the absence of 0.3 g/L LiCl. The TBF of Hydra fed with *Artemia* prepared in the specified salt solution was examined within 10 hr after trypsin treatment (crystallized, porcine pancreas, Wako Pure Chem., Osaka, Japan; 100 pg/ml, 10 min) at GSM concentrations of 0.1 and 10 µM as described below. In the course of this experiment, Hydra of mass culture were fed with *Artemia* that had been hatched in the common salt solution without any supplements.

Examination of conditions of trypsin treatment

Hydra were treated with trypsin at a concentration specified for 10 min in the buffer, 1 mM HEPES, 1 mM NaHCO₃, and 1 mM CaCl₂ (pH 7.7) (HEPES-buffered BC, Hanai et al., 1995). After the treatment, Hydra were kept in 1 mM NaHCO₃, 1 mM CaCl₂ after a brief rinse until subject to the behavioral assay. After a specified period of time, 10 Hydra were transferred in a 35 mm dish containing 2 ml of PIPES solution (1 mM PIPES, 1 mM CaCl₂, pH 6.2), and the response to 10 µM GSM was examined in the presence and the absence of 1 pg aFGF (TOYOBO, Osaka, Japan) as described below.

Examination of the TBF

After a rinse, 10 Hydra were transferred in the 2 ml of PIPES solution (1 mM PIPES, 1 mM CaCl₂, pH 6.2) containing a test peptide in 1 µl of 0.2% PRIONEX (Merck, Darmstadt, Germany). The dish was placed on the stage of a binocular microscope, which was kept at 20 °C by circulating temperature-regulated water. After 5 min, a small amount (<10 µl) of concentrated GSM solution was applied to stimulate Hydra to a final concentration of 0.1, 0.3, 3, 10, and 50 µM. At these stimulant concentrations, we observed the largest suppression by various biologically active substances (Hanai et al., 1987). After a gentle swirl, the response was observed with the binocular microscope (x8); the number of Hydra that showed TBF was counted at each minute for 10 min. The response was sum of Hydra exhibiting TBF each minute from 6 to 10 min divided by the total number of Hydra. (Hanai and Matsuoka, 1995).

Rat CSF sampling

Eight-week old male Sprague-Dawley rats (Japan Charles River, Yokohama, Japan) were kept in a temperature (22 ± 2 °C) and humidity-controlled environment on 12:12 light-dark cycle (on: 1800-0600). Food and water were available *ad libitum*. Rats were fasted overnight the day before the experiment. The rats were anesthetized with pentobarbital and CSF was

collected from the cisterna magna.

All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985).

Development of modulation by biologically active peptides on the TBF

Tested peptides were aFGF, basic fibroblast growth factor, platelet-derived growth factor (Becton Dickinson Labware, Bedford, MA), calcitonin gene related peptide (human), cholecystokinin tetrapeptide (30-33), cholecystokinin octapeptide (26-33, non-sulfated form), corticotropin releasing factor (CRF, human), growth hormone releasing factor (human), neuropeptide Y (human), and substance P (human). All synthetic peptides were products of Peptide Institute Inc. (Osaka, Japan).

The effect of TGF- β on the TBF

CRF was used to suppress the TBF because of its availability and stable suppressive effect at the highest GSM concentration (50 μ M). Mixtures of CRF (1 ng) and TGF- β 1, -2 or -3 (1 ng) were added to the Hydra assay medium, and the TBF was observed at 50 μ M GSM concentration.

The effect of active TGF- β 1 or latent TGF- β 1 on the TBF

Latent TGF- β 1 (recombinant human latent TGF- β 1, R&D Systems, MN, USA) (1 ng) or active TGF- β 1 (1 ng) was added to the Hydra assay medium, and the TBF was observed at each of the five GSM concentrations. When suppression-eliminating effect was examined at all stimulating conditions, we used 1 μ l of rat CSF diluted to 10^4 with 0.2 % PRIONEX solution as a suppressor of the TBF. Rat CSF, which would contain various biologically active substances, was strongly suppressive at each of the five GSM concentrations as observed previously (Hanai et al., 1989; Manabe et al., 1999 manuscript in preparation). A mixture of latent TGF- β 1 or active TGF- β 1 and diluted rat CSF was also examined.

The effect of members of TGF- β superfamily on the TBF

I
We examined the effects of TGF- β 1, BMP4 (recombinant, *Xenopus laevis*, a kind gift from Dr. Naoto Ueno) (Nishimatsu et al., 1992), GDNF (human glial derived neurotrophic factor, Alomone Labs Ltd., Jerusalem, Israel), inhibin (human follicular fluid, Biogenesis Ltd., Poole, England), and activin A (recombinant bovine activin A, Innogenetics NV., Zwijndrecht, Belgium). The stock solutions of these peptides were prepared according to the manufacture's directions, and used after dilution with 0.2 % PRIONEX. Peptides (1 ng) were added to the Hydra assay medium individually, and the TBF were observed at the each of five GSM concentrations.

Statistics

All statistical tests were done with use of StatView (SAS Institute Inc., Cary, NC).

Results

Effect of metal ions in Artemia-hatching solution on the TBF

In preliminary experiments, we observed that the salt composition, in which solution *Artemia* had been hatched, affected the TBF of Hydra: The common salt from Japan Tabacco was better than the reagent grade NaCl. Then we assumed that minor heat-stable components contained in the salt had a good effect on the TBF. Metal salts, which were contained in the biological tissues as cofactors for transport proteins and enzymes, were examined.

Various salts were included into the *Artemia* hatching solution. Multivalent cations such as Fe^{3+} or Zn^{2+} effectively potentiated the TBF in response to $0.1 \mu\text{M}$ GSM (Fig. 1). Similar capability of these ions was observed in the response to higher concentrations of GSM, though the effect was less apparent (data not shown). This effect was stronger when LiCl was also included especially at lower concentration of ZnCl_2 (Fig. 2). After extensive examinations, 1 ng/L ZnCl_2 and 0.3 g/L LiCl were included in the common salt. Hydra fed with *Artemia* hatched in the solution containing FeCl_3 tended to show the TBF less modulated by biologically active substances (unpublished observation).

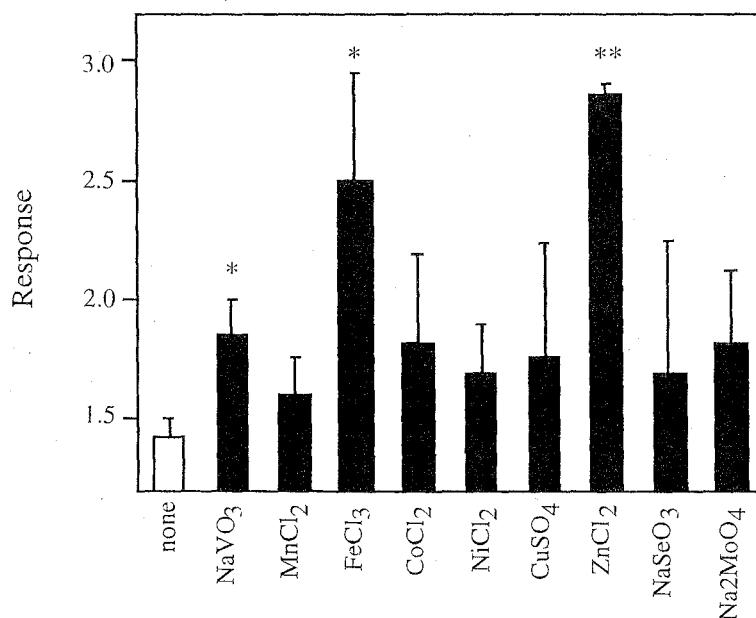


Fig. 1 Effect of various metal ions included in the *Artemia* hatching solution on the response of TBF of Hydra. Specified metal compounds (10 ng/L) were included in the *Artemia* hatching solution (30 g/L common salt and 0.3 g/L LiCl). Hydra was fed with *Artemia* 2 times every other day before tests: none, control response of hydra, which was fed with *Artemia* hatched in the common salt solution (30 g/L) without any supplement. Hydra were stimulated by $0.1 \mu\text{M}$ GSM. Values are means \pm S.D. ($n=3-7$). Statistically significant from none by Dunnet test (*; $p<0.05$, **; $p<0.01$).

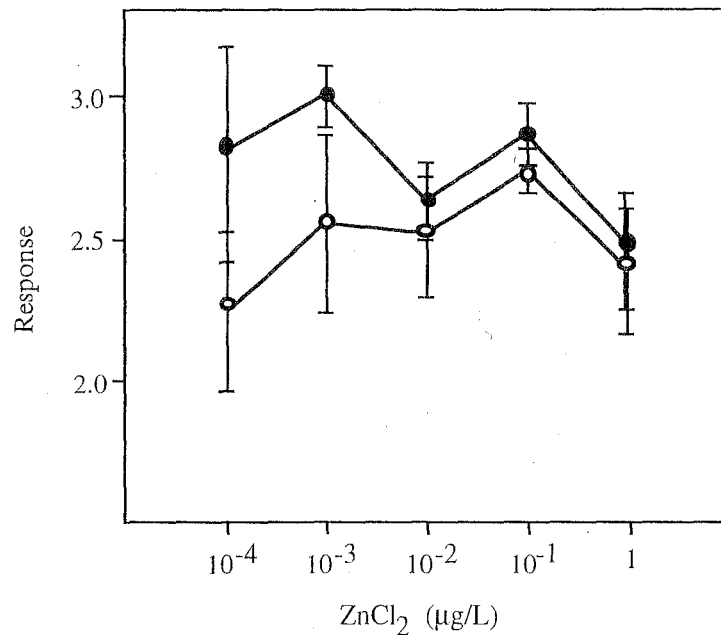


Fig. 2 Effect of ZnCl_2 and LiCl included in the *Artemia* hatching solution on tentacle ball formation. The concentrations of ZnCl_2 were varied in the presence of 0.3 g/L LiCl (closed circle) or in the absence (open circle). Hydra were stimulated at 10 μM GSM. The vertical axis is the response value. Values are means \pm S.D. (n=6-8). The group of GSM response in the presence of LiCl is significantly different from that in the absence of LiCl ($p < 0.05$, ANOVA, difference of any pair at the same ZnCl_2 concentration was not significant by multiple comparisons).

Development of modulation by biologically active peptides on the TBF

When Hydra were treated with trypsin at concentrations lower than 1 $\mu\text{g}/\text{ml}$ for 10 min, Hydra developed a new repertory of glutathione-induced behavior, TBF as reported earlier (Hanai, 1998; Hanai and Matsuoka, 1995). When the concentration of trypsin was 100 pg/ml , this response was sensitively suppressed by acidic fibroblast growth factor for successive more than 8 hours (Table 1). When trypsin concentration was 10 pg/ml , the onset of the suppression retarded a few hours. On the contrary, when the trypsin concentration was 1 ng/ml , the suppression was observed for the first few hours, after that the suppression disappeared.

We examined the TBF at each of the 5 concentrations in the presence of platelet-derived growth factor, aFGF, and basic fibroblast growth factor (Fig. 3). Essentially the same suppression was observed for trypsin treated Hydra in the presence of these factors as reported earlier in which experiments any treatment of Hydra such as trypsin treatment, was not required to observe the TBF because of unknown environmental reasons (Hanai et al., 1987; Hanai et al., 1989). Then the sensitivities to the biologically active substances developed by the trypsin treatment appear to be equivalent to that observed previously without any treatment.

We also examined the effect of some synthetic peptides on the TBF. Figure 4 shows the suppression for calcitonin gene related peptide, cholecystokinin peptides, CRF, growth

hormone releasing factor, neuropeptide Y, and substance P. The TBF was examined in the presence of one of these peptides at a concentration of 0.5 ng/ml. The minimum concentration of the peptide at which the suppression was observed was 0.5 fg/ml for CRF. The TBF in the presence of synthetic peptides also confirms that specific suppression occurs only on the response stimulated by specific stimulatory GSM concentrations depending on each of individual peptides.

Table 1 Development of factor-sensitivity of the response in Hydra potentiated by trypsin

Response					
+aFGF			control		
h	Average \pm SD	n	Average \pm SD	n	
potentiated by 10 pg/ml trypsin					
2	3.52 \pm 0.77	5	3.58 \pm 0.17	4	
4	3.30 \pm 0.48	5	3.60 \pm 0.52	4	
6	1.62 \pm 0.63	5	3.35 \pm 0.37	4	p<0.05
8	1.38 \pm 0.51	5	3.23 \pm 0.24	4	p<0.01
potentiated by 100 pg/ml trypsin					
0.5	2.80 \pm 0.41	4	3.58 \pm 0.17	4	p<0.05
1	1.86 \pm 0.21	5	3.88 \pm 0.25	4	p<0.01
2	1.86 \pm 0.67	5	3.48 \pm 0.25	4	p<0.05
5	1.84 \pm 0.40	5	3.55 \pm 0.21	4	p<0.05
8	1.60 \pm 0.33	5	3.30 \pm 0.08	4	p<0.01
potentiated by 1 ng/ml trypsin					
0.5	2.68 \pm 0.64	5	3.63 \pm 0.32	3	
1	1.98 \pm 0.37	5	3.53 \pm 0.48	4	p<0.01
3	2.38 \pm 1.00	5	3.30 \pm 0.24	4	
5	3.26 \pm 0.67	5	3.48 \pm 0.49	4	
8	3.42 \pm 0.51	5	3.70 \pm 0.32	4	

Statistical analysis was paired t-test (2 tailed). Animals were treated with trypsin at specified concentrations for 5 min in BC solution, and then they were kept in BC solution without trypsin for specified time (h). Animals were stimulated with 10 μ M GSM in the presence of 0.1 pg/ml aFGF (+aFGF) or in the absence of aFGF (control).

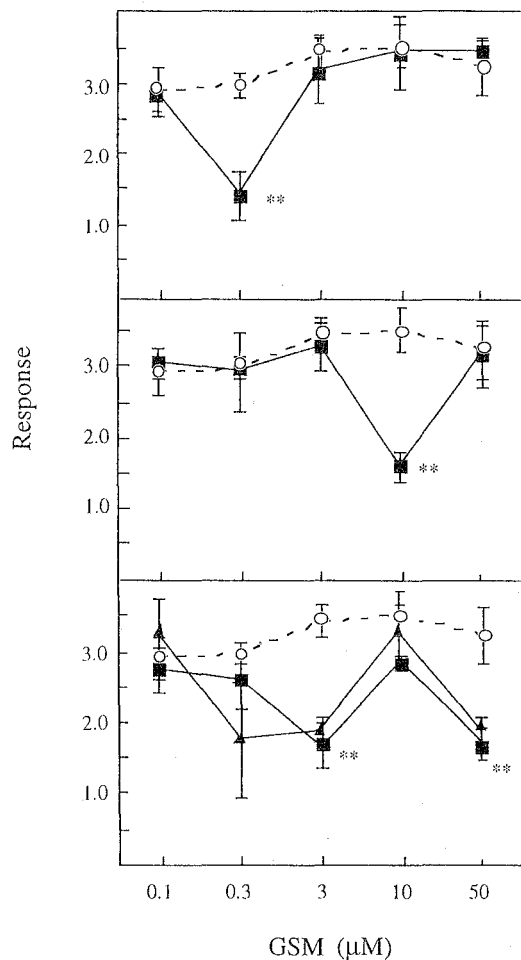
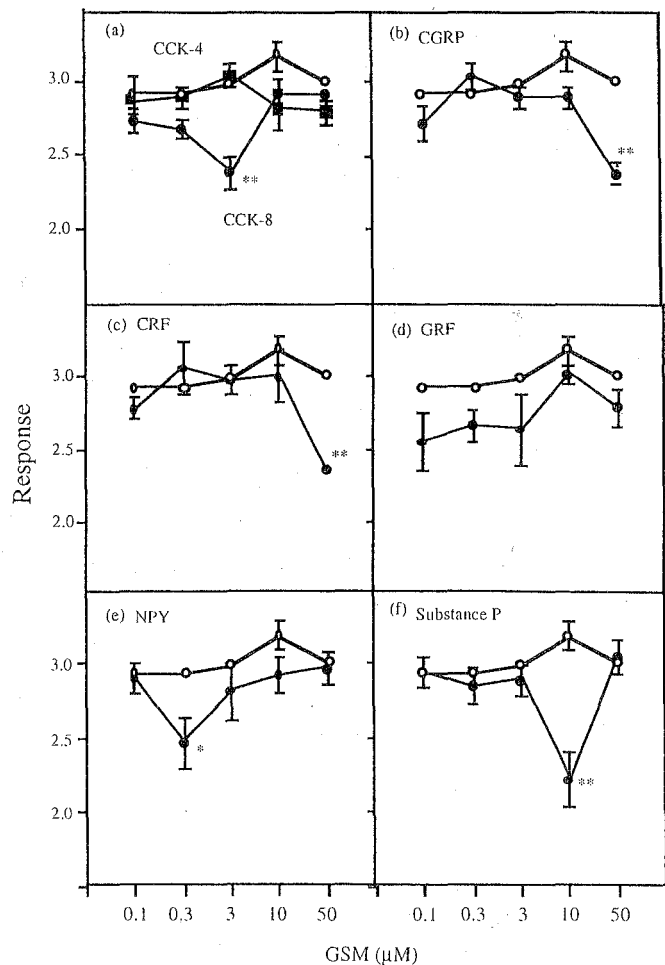


Fig. 3 Effect of growth factors on tentacle ball formation potentiated by trypsin. (Top) Hydra was stimulated in the presence of PDGF (0.5 ng/ml, filled square): (Middle) aFGF (0.5 pg/ml, filled square): (Bottom) basic FGF (filled square, 0.05 pg/ml; filled triangle, 0.5 ng/ml). Open circles on all figures are the control response values (the response in the absence of growth factors). The horizontal axis is the GSM concentrations used to stimulate hydra. The vertical axis is the response. Values are means \pm S.D. (n=4). Statistically significant from control response by Student's t test (**; $p < 0.01$).

Fig. 4 The response in the presence of various synthetic peptides. (a) Cholesystochinin-tetrapeptide, octapeptide (CCK-4, filled square; CCK-8, filled circle): (b) calcitonin gene related peptide (CGRP, filled circle): (c) corticotropin releasing factor (CRF, filled circle): (d) growth hormone releasing factor (GRF, filled circle): (e) neuropeptide Y (NPY, filled circle): (f) substance P (filled circle). The concentration of individual peptide was 0.5 ng/ml. Vertical axis is the response. Open circles on the dotted line are values of control response, which were examined in the absence of peptide. Values are means \pm S.D. (n=5). Statistically significant from control response by Student's t test (*; $p < 0.05$, ** $p < 0.01$).



The effect of TGF- β on the TBF

CRF suppressed the response to 50 μ M GSM (Fig. 5). But when TGF- β 2 was included in the medium at the same time, this suppression by CRF was eliminated. Three distinct forms of TGF- β have been identified in mammals (TGF- β 1, 2, 3) (Massague, 1990). The suppression by CRF was eliminated to a similar extent by TGF- β 1, TGF- β 2 or TGF- β 3, and no difference in suppression-eliminating effects was observed among TGF- β isoforms (Fig. 5).

We examined the suppression-eliminating effect of TGF- β 1 on other stimulating conditions. This time, we used the rat CSF as a suppressor, because CSF was suppressive on all the stimulatory conditions (Hanai et al., 1989; Manabe et al., 1999 manuscript in preparation). Different TGF- β 1 concentrations were required for the suppression-eliminating effect to occur depending on the stimulating conditions (Fig. 6): >200 fM for the response at 0.1 μ M GSM; >20 fM at 0.3 μ M GSM; >0.2 fM at 3 μ M GSM; >20 fM at 10 μ M GSM; >0.06 fM at 50 μ M GSM. The response to 50 μ M GSM was the most sensitive to TGF- β 1.

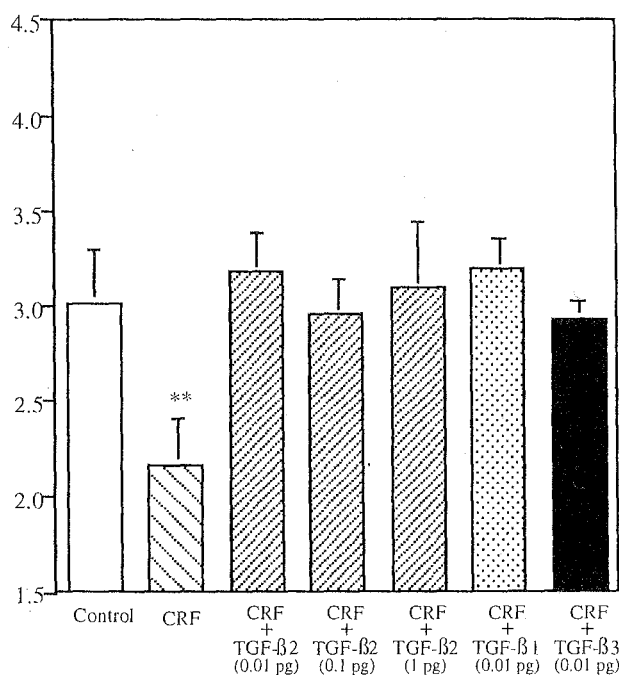


Fig. 5 The suppression-eliminating effect of isoforms of TGF- β on the CRF suppression of the GSM response at 50 μ M GSM. TGF- β 1, -2 and -3 isoforms produced similar suppression-eliminating effects on the suppression by CRF. Control response was observed in the presence of the diluent, 0.2% PRIONEX (1 μ l). Values are means \pm S.D.(n=5). **, Significantly different from control by Dunnet test ($p < 0.01$).

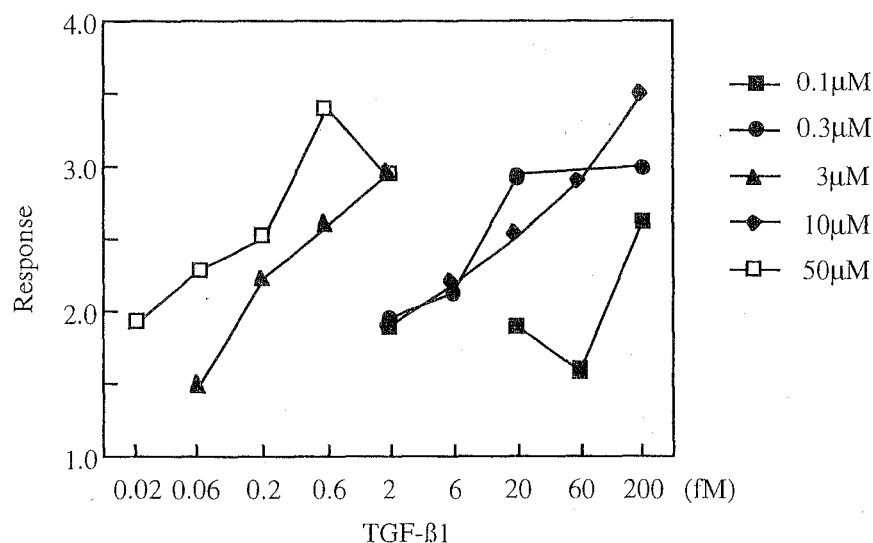


Fig. 6 Concentration-dependent effect of TGF-β1 at each of the five GSM concentrations (representative result). The GSM response was examined in the presence of rat CSF and a specified concentration of TGF-β1. Elimination of GSM response suppression was observed at concentrations of TGF-β higher than 200 fM (0.1 μM GSM), 20 fM (0.3 μM GSM), 0.2 fM (3 μM GSM), 20 fM (10 μM GSM) and 0.06 fM (50 μM GSM).

The effect of active TGF-β1 or latent TGF-β1 on the TBF

Latent TGF-β1 did not suppress the TBF as active TGF-β1 did (Fig. 7). To examine the suppression-eliminating effect of latent TGF-β1, we studied the mixture of latent TGF-β1 and rat CSF. When the TBF was examined in the presence of a mixture of active TGF-β1 and rat CSF, the suppression by CSF was greatly reduced (Fig. 8), while latent TGF-β1 was not able to eliminate the TBF suppression caused by rat CSF, indicating that latent TGF-β1 was ineffective. Apparent stronger suppression on 0.3 μM TBF in the presence of CSF and latent TGF-β1 (Fig. 8) may be an artifact derived from different batches of CSF samples in the control experiment.

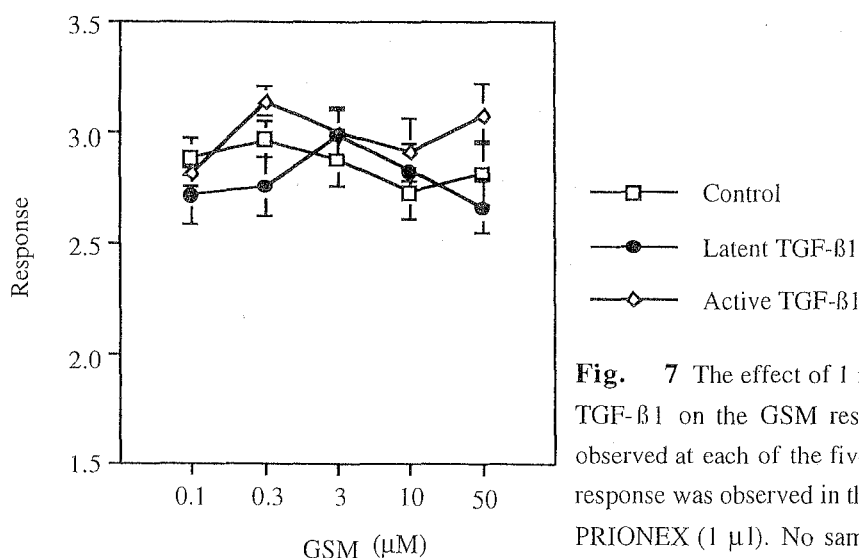


Fig. 7 The effect of 1 ng latent TGF-β1 or 1 ng active TGF-β1 on the GSM response. The GSM response was observed at each of the five GSM concentrations. Control response was observed in the presence of the diluent, 0.2% PRIONEX (1 μl). No samples had an effect on the GSM response. Values are means ± SEM (n=6-8).

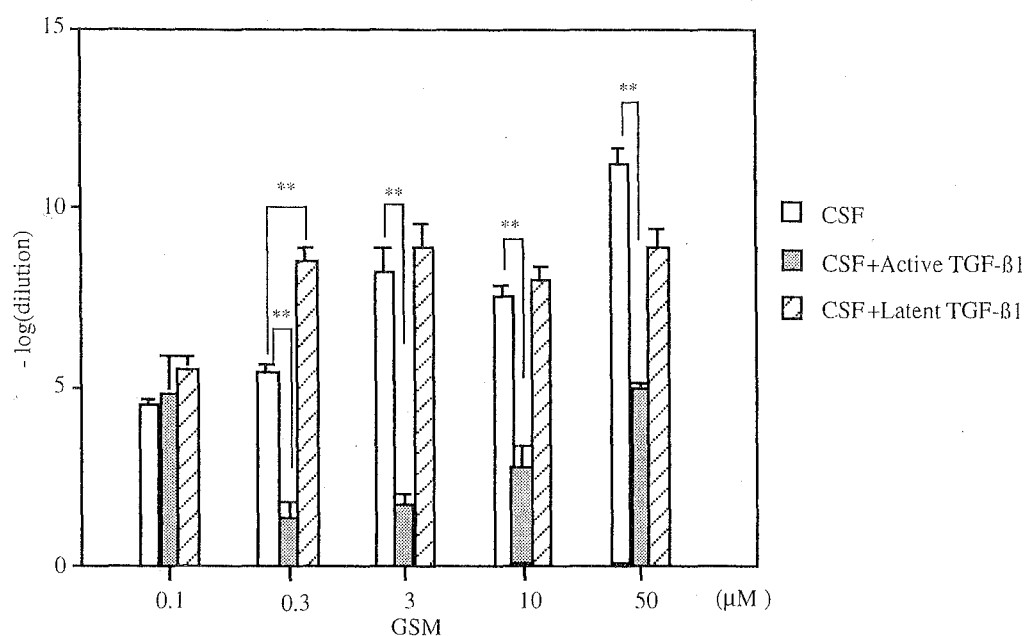


Fig. 8 The suppressive activity of CSF, CSF+active TGF-β1 (10 pg), CSF+latent TGF-β1 (1 ng) on the GSM response. The suppressive activity was expressed as the maximum dilution at which the suppression of the GSM response was observed. CSF+latent TGF-β1 gave a similar response to CSF alone. Values are means ± SEM (CSF; n=9, active TGF-β1; n=6, latent TGF-β1; n=5). **: Significantly different from CSF by Dunnet test ($p < 0.01$).

The effect of members of TGF-β superfamily on the TBF

TGF-β alone did not show the suppressive effect on all the TBF examined, while TGF-β superfamily peptides showed the suppressive effect, that was totally different from the effect of TGF-β1 (Fig. 9). The suppression by these peptides was observed at concentrations higher than 5.8 fM (BMP4), 1.7 pM and 0.17 fM (GDNF, 3 μM GSM and 10 μM GSM

respectively), 0.2 aM (activin), 1.6×10^{-3} aM (inhibin) (Fig. 10). None of TGF- β superfamily peptides showed the ability to eliminate the suppression of TBF by rat CSF (data not shown). It appears unlikely that a peptide suppresses a component of TBF and the same peptide eliminates the suppression of other components of TBF by other peptides.

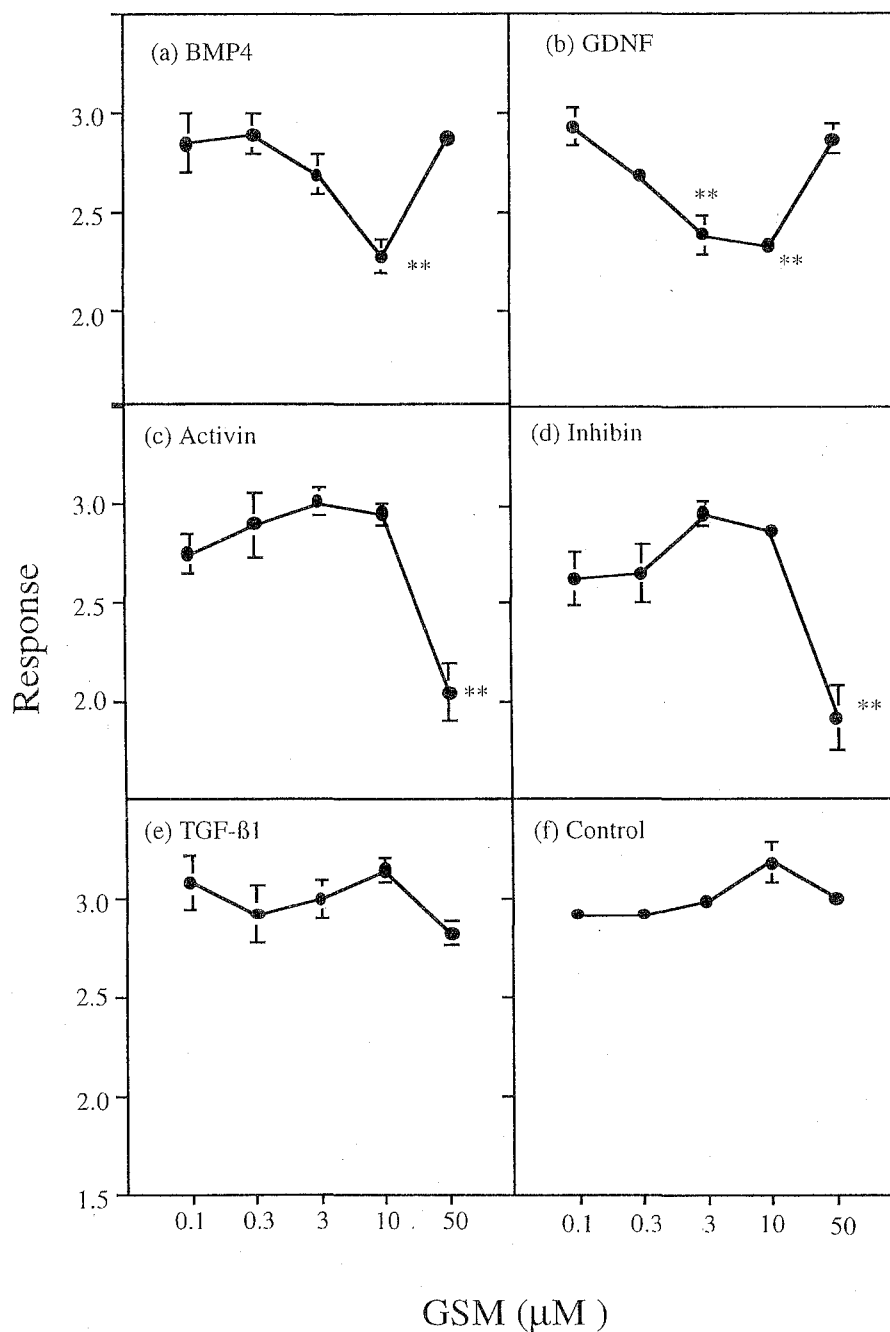


Fig. 9 The TBF in the presence of a member of the TGF- β superfamily (1 ng). (a) BMP4 (b) GDNF (c) activin (d) inhibin (e) TGF- β 1 (f) control. The response was observed at each of the five GSM concentrations specified in this figure. Control response was observed in the presence of the diluent, 0.2% PRIONEX (1 μ l) alone. Except for TGF- β 1, members of TGF- β superfamily suppressed the TBF at the specified GSM concentrations. Vertical axis is response, and horizontal axis is GSM concentration. Values are means \pm SEM (n=5-6). **: Significantly different from control response by Student's t test ($p < 0.01$).

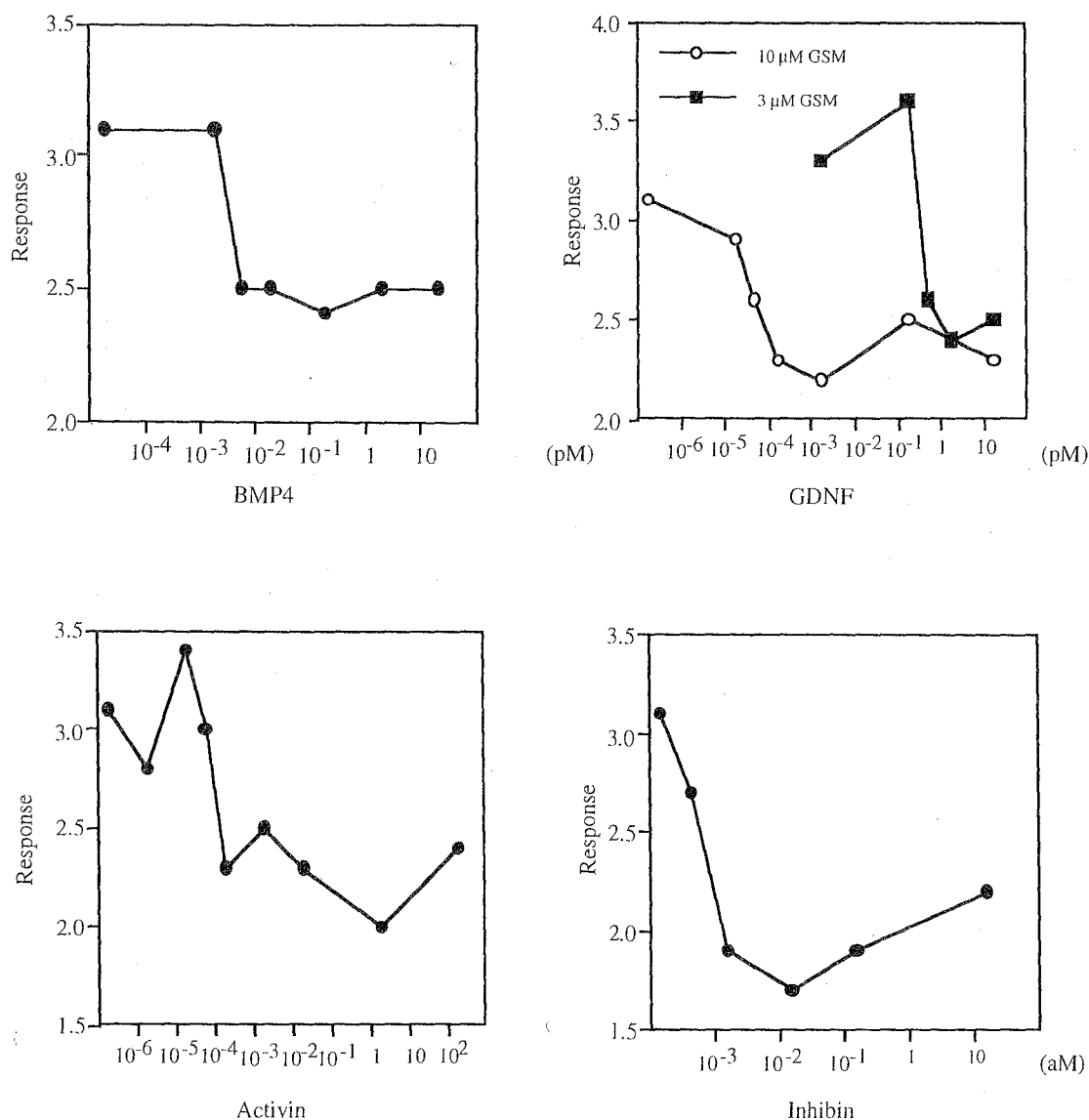


Fig. 10 The effect of BMP4, GDNF, activin and inhibin at different concentrations on TBF. BMP4; suppression of the TBF at 10 μ M GSM was observed at BMP4 concentrations greater than 5.8 fM. GDNF; suppression of the TBF at 3 μ M and 10 μ M GSM was observed at GDNF concentrations higher than 1.7 fM and 0.17 fM, respectively. Activin; suppression of the TBF at 50 μ M GSM was observed at activin concentrations higher than 0.2 aM. Inhibin; suppression of the TBF at 50 μ M GSM was observed at inhibin concentrations higher than 1.6×10^{-3} aM.

Discussion

In this study, the conditions for the TBF were examined in detail. Hydra, which fed with *Artemia* hatched in the salt solution supplemented with ZnCl_2 , showed the strong TBF after the trypsin treatment. The TBF was modulated by a number of biologically active peptides under an appropriate condition of the trypsin treatment.

Direct inclusion of ZnCl_2 into the culturing medium was poisonous to Hydra at higher concentrations or ineffective at lower concentrations (unpublished observations). It was effective only when ZnCl_2 was given through food though we did not examine ZnCl_2 levels in *Artemia*. It is noteworthy that Zn deficiency leads to hypogeusia in human (Atkin-Thor et al., 1978), though no reason is conceivable for an apparent common requirement of Zn both in human and Hydra at present. A Zn-metalloprotein, gustin, from human parotid is reported to be carbonic anhydrase IV, and to correlate the loss of taste (Thatcher et al., 1998). It is also interesting to note that incubation of Hydra with bicarbonate solution after trypsin treatment is important to observe TBF (unpublished observation).

A lot of biologically active substances such as nitric oxide (Colasanti 1995; Colasanti, 1997), arachidonic acid and eicosanoids (Pierobon, 1997) have been reported to modulate the feeding response of Hydra. The effect of peptides on the TBF is outstanding: Large number of biologically active peptides may suppress the TBF in a specific way depending on individual peptides. Other peptides that were not examined in this study would also suppress the TBF. On the other hand, the low molecular weight classical transmitter substances, such as acetylcholine (Erzen and Brzin, 1978), biogenic amines (Hanai et al., 1984; Ventulini, 1992), and γ -aminobutyric acid (Concas, 1998) have weak or no effect on the TBF. It appears that Hydra, one of the animals with the most primitive nervous system, extensively uses peptides as information-transmitting substances. Hydra nervous system has been visualized by a number of antibodies to biologically active peptides (Grimmelikhuijzen and Westafall, 1995). Takahashi et al. (1997) reported abundant peptides from Hydra tissues that were, potentially, biologically active.

The suppression-eliminating effect of TGF- β was unique among peptides examined. None of peptides examined including members of TGF- β superfamily showed the similar effect. TGF- β isoforms have diverse biological activities including the regulation of cell proliferation and differentiation, stimulation of matrix formation, regulation of the cell migration and stimulation of adhesion molecule expression (Massague et al., 1992). Furthermore, TGF- β plays an important role as a modulator of the immune reaction, a mediator of tissue repair in bone formation and remodeling and processing of wound healing (Barnard et al., 1990; Clark et al., 1998; Grande, 1997; O'Kane et al., 1997). At present, we do not understand why mammalian TGF- β causes an effect on the TBF. It is likely that the Hydra, one of the most primitive organisms, has a system influenced by primordial TGF- β . This unique effect of

TGF- β may be related to a mechanism involved in the suppression of the TBF as well as the possible unique structural feature of TGF- β itself (Schlunegger et al., 1992).

The sensitive, specific modulation of the TBF by biologically active peptides would be useful for the study of these peptides in biological samples.

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CHAPTER II Identification and determination of transforming growth factor-beta like activity in the rat cerebrospinal fluid after exhaustive exercise

Introduction

Fatigue is an important sensation for animals to alert the depletion of energy and the need of the body to rest. At present, the cause of central fatigue has been proposed to be related to the change in monoamines (serotonin, acetylcholine and dopamine) (central fatigue hypothesis; Bailey et al., 1992; Bailey et al., 1993; Blomstrand et al., 1988; Blomstrand et al., 1989; Davis et al., 1997; Jakeman, 1998; Newsholme et al., 1991). Elevated free tryptophan in plasma, the precursor of serotonin, during exercise is proposed to result in the increase of serotonin synthesis in the brain, which causes the sensation of fatigue. However, several observations against this hypothesis have been reported (Pannier et al., 1995; Stensrud et al., 1992; van et al., 1995). Recently we reported that the injection of high molecular weight fraction of the cerebrospinal fluid (CSF) from rats after the exhaustive physical exercise into the brains of sedentary mice decreased the spontaneous motor activity (Inoue et al., 1998), indicating that a high molecular weight component also may be responsible for the central fatigue. Furthermore, the result in chapter I and all of our studies including the hydra behavioral tests as well as the mice behavioral tests suggest that the high molecular weight substance was TGF- β (Inoue et al., 1999).

In the hydra behavioral tests, we observed the tentacle ball formation (TBF), a component of the feeding response of hydra, to detect biologically active substances such as cytokines and growth factors in the CSF. TBF was elicited at five different concentrations of S-methyl-glutathione (GSM). Many biologically active substances modulated the TBF differently in the presence of GSM at these five concentrations (Hanai et al., 1987; Hanai et al., 1989). Then, by observing these modulating patterns, we can obtain information about the unknown substances in samples containing various peptides (Hanai, et al., 1984; Hanai et al., 1987; Hanai, et al., 1989; Torii et al., 1993). TBF was modified by many biologically active peptides at low concentrations but not by neurotransmitters. Further, the effect of most peptides on TBF is suppressive, but that of TGF- β was quite different (chapter I). TGF- β nullified the suppressive effect of other peptides on TBF.

In this study, we focused on the unique effect of TGF- β and examined the amount of TGF- β in the CSF using the hydra bioassay, for a detailed investigation of TGF- β -like activity in CSF after exhaustive exercise.

Materials and Methods

Hydra culture and behavioral test

Hydras (*Hydra japonica*) were cultured as described chapter I and were pretreated with trypsin to potentiate TBF (Hanai et al., 1995 and chapter I).

TBF was assayed as describe chapter 1.

CSF sampling

Eight-week old male Sprague-Dawley rats (Japan Charles River, Yokohama, Japan) were kept in a temperature (22 ± 2 °C) and humidity-controlled environment on a 12 h light/12 h dark cycle (lights on at 18:00). Food and water were available *ad libitum* until the day before the experiment, when the rats were fasted overnight. For collection of CSF from fatigued rats (Fatg CSF), rats were subjected to a series of 8 sessions of swimming (15 min per session), each separated by a 5-min resting period, in a forced-swimming apparatus (a current pool) at a current of 10 l/min (Matsumoto et al., 1996). A weight (2% of the body weight) was attached to the tail from the second session. The rats were anesthetized with pentobarbital and CSF was collected from the cisterna magna within 5 min of completing the final exercise session. Control CSF was collected from sedentary rats (Sed CSF).

All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985).

TBF to Sed CSF, Fatg CSF, Sed CSF+Fatg CSF and Sed CSF+TGF- β 1

The above-mentioned CSF samples, an equal mixture of Sed CSF and Fatg CSF, and a mixture of Sed CSF (1 μ l) and TGF- β 1 (10 pg) were subjected to the Hydra behavioral test at 5 different GSM concentrations. When TBF was suppressed by the original sample, it was diluted with 0.2% PRIONEX until the effect disappeared.

The effect of the mixture of anti-TGF- β IgG and Fatg CSF on TBF

Fatg CSF (450 μ l) was incubated with the pan-specific anti-TGF- β IgG (R&D Systems, MN, USA) (4.8 μ g) for 1 hr on ice. Normal rabbit IgG (R&D Systems, MN, USA) (4.8 μ g) was used instead of the anti-TGF- β IgG in the control experiments. Then, protein A beads (Affigel Protein A, Bio Rad, CA, USA) (4.8 μ l) were added to this solution. The antigen-IgG complex was bound to the beads and was precipitated by centrifugation for 5 sec (10000 rpm). The supernatant was subjected to the hydra assay.

Quantitative analysis

- (1) The standard relationship between TGF- β and anti-TGF- β IgG

Corticotropin-releasing factor (CRF; human, rat: Peptide Institute, Osaka, Japan) was used to suppress the TBF. The TBF at 50 μ M GSM was examined in the following way. The mixture (total volume 30 μ l) of CRF (1 fg/ μ l) in 10 μ l, designated amount of TGF- β 1, -2 or -3 (R&D Systems, MN, USA) in 10 μ l, and an excess amount of the anti-TGF- β IgG (initial concentration 1.24 pg /10 μ l) was preincubated for 10 min on ice. Then 3 μ l of the mixture was added to the hydra test solution (the PIPES solution). After 5 min of incubation, GSM was applied at a final concentration of 50 μ M and the TBF was observed. This test was repeated with the reducing amount of anti-TGF- β IgG maintaining the same amount of CRF and TGF- β until the suppression of TBF by CRF was nullified by TGF- β . In this way, we determined the minimum amount of anti-TGF- β IgG necessary to overcome the nullifying effect of TGF- β on suppressed TBF by CRF. TGF- β 2, - β 3 at 100, 10, 1, 0.1 and 0.01 fg and TGF- β 1 at 300, 30, 3, 0.3 and 0.03 fg were used in this experiment.

(2) Measurement of TGF- β in the CSF

CSF (400 μ l) was ultrafiltrated using Microcon-10 (Amicon, Massachusetts, USA) to prepare the high-molecular-weight fraction. The retained fraction on the membrane was retrieved in 0.1 % BSA/saline (200 μ l) and diluted x100 with the 0.2% PRIONEX solution. A mixture of the diluted sample (10 μ l) and the pan-specific anti-TGF- β IgG (124 ng) was subjected to the hydra assay after incubation on ice for 10 minutes. When the suppression of TBF was observed, the amount of anti-TGF- β IgG was reduced and TBF was re-examined. This process was repeated until the suppression of TBF was nullified. In this way, we determined the minimum amount of anti-TGF- β IgG necessary to suppress TBF. From this value we estimated the amount of TGF- β in the CSF samples using the relationship between the amount of anti-TGF- β IgG and TGF- β determined in the above section (quantitative analysis).

Statistics

Statistical significance was analyzed by the one-way analysis of variance (ANOVA). Groups were compared by multiple comparisons (Tukey-Kramer test).

Results

TBF to Sed CSF, Fatg CSF, Sed CSF+Fatg CSF and Sed CSF+TGF- β 1

The suppressive effect of Sed CSF on the TBF induced by GSM 3, 10, and 50 μ M was more potent than that of Fatg CSF. The effects of the mixture of Sed CSF and Fatg CSF and the mixture of Sed CSF and TGF- β 1 were similar to that of Fatg CSF (Fig. 1). These results suggest that Fatg CSF contains active TGF- β , eliminates the suppression of TBF by biologically active substances, suggesting that active TGF- β appears in CSF after exhaustive exercise (chapter I).

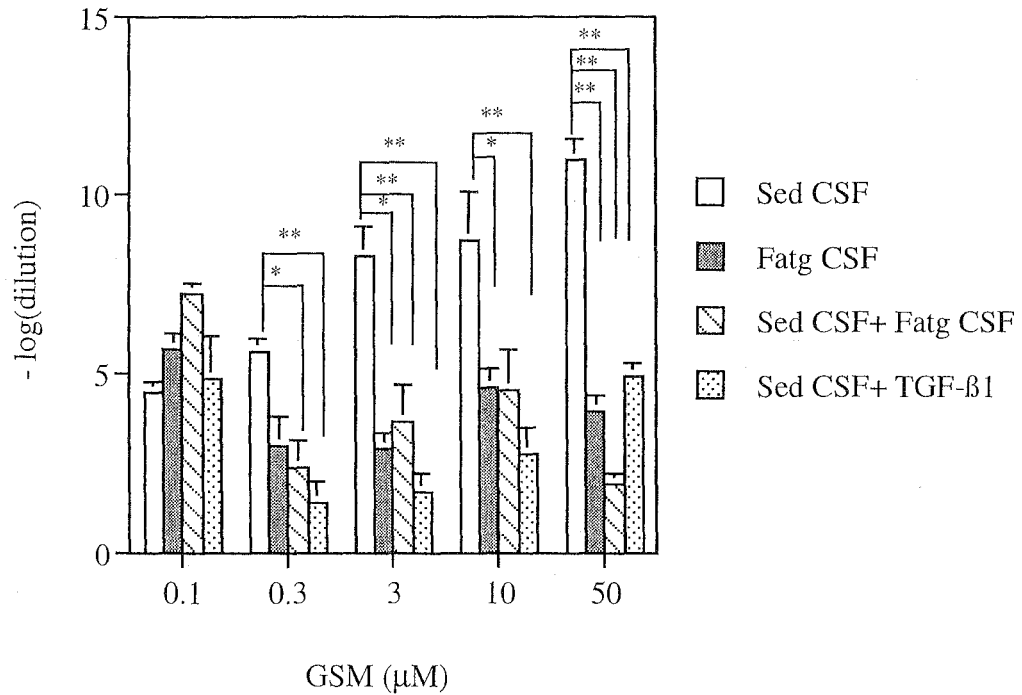


Fig. 1 Effects of sedentary rat CSF (Sed CSF), fatigued rat CSF (Fatg CSF), a mixture of Sed CSF and Fatg CSF, and a mixture of Sed CSF with TGF-β1 (10 pg) on TBF. The vertical axis is the logarithm of the maximum dilution at which suppression of TBF was observed. The higher bar indicates stronger suppression of TBF. Values are means ± SEM (Sed CSF; n=9, Fatg CSF; n=10, Sed CSF+Fatg CSF; n=4, Sed CSF+TGF-β1; n=6). Significantly different from sedentary rat CSF (**; $p < 0.01$, *; $p < 0.05$).

The effect of the mixture of the anti-TGF-β IgG and Fatg CSF on TBF

Sed CSF suppressed TBF more strongly than Fatg CSF, but the difference was nullified by the addition of anti-TGF-β IgG to the Fatg CSF (Fig. 2). Control IgG did not show this effect. This result is another line of evidence supporting our previous conclusion that TGF-like activity in the CSF increased after physical exercise (Inoue, et al., 1999). Thus, we quantitatively examined the amount of the TGF-β-like activity in the Fatg CSF using anti-TGF-β IgG.

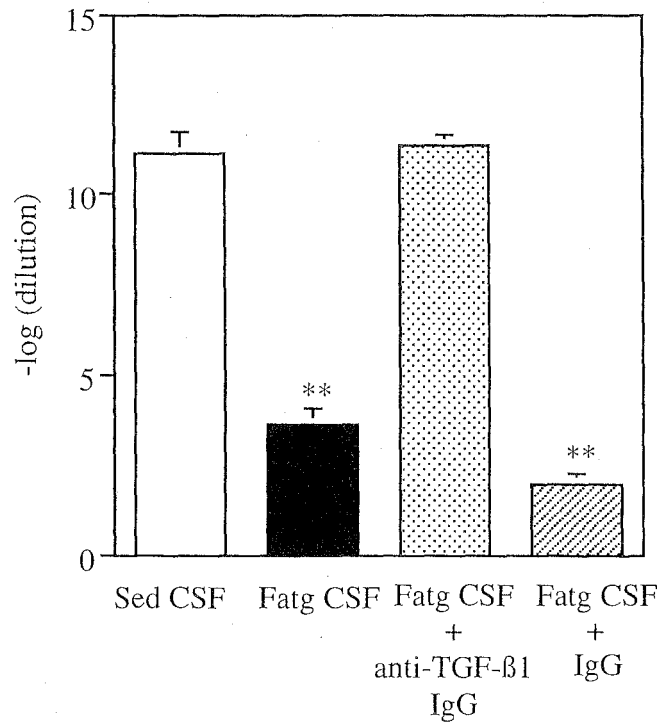


Fig. 2 Suppression of TBF by Sed CSF and that by Fatg CSF with and without incubation with anti-TGF- β IgG. Fatg CSF incubated with anti-TGF- β IgG showed the suppressive activity nearly equal to that of Sed CSF. Suppression of the TBF was examined after stimulation by 50 μ M GSM. The vertical axis is $-\log$ of the maximum dilution (suppression of TBF). Values are means \pm S.D. (Sed CSF, $n=9$; Fatg CSF, $n=10$; Fatg CSF treated with pan-specific anti-TGF- β IgG, $n=5$; Fatg CSF treated with control IgG, $n=5$). **, Significantly different from Sed CSF ($p<0.01$).

Quantitative analysis

First, we examined the TBF of hydra in the mixture containing 1 fg CRF, 0.01 fg TGF- β 2 and various amounts of anti-TGF- β IgG, by the method described in Materials and Methods (Fig. 3). TBF was not suppressed by 0.03 pg or smaller amount of anti-TGF- β IgG. This means that 0.037 pg anti-TGF- β IgG made 0.01 fg TGF- β inactive in this system. By the same method, the minimum amount of TGF- β IgG necessary to inactivate various amounts of TGF- β 1, - β 2 and - β 3 in this system were examined. A linear relationship (correlation coefficient 0.99) was observed between the amounts of TGF- β 1, - β 2 and - β 3 and the amount of anti-TGF- β IgG necessary to inactivate them, over a wide range of TGF- β concentrations from 0.01 to 100 fg/ml (Fig. 4).

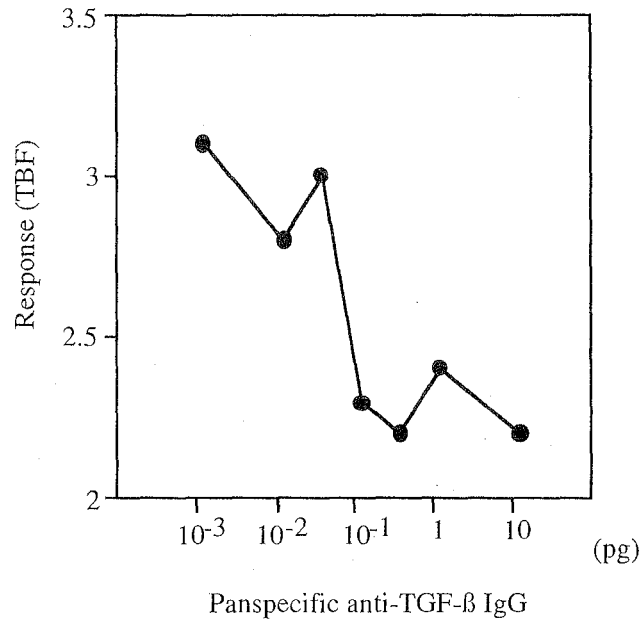


Fig. 3 Effect of the pan-specific anti-TGF-β IgG on the TBF in the presence of 1 fg CRF and 0.01 fg TGF-β. The TBF elicited by stimulation with 50 μM GSM was examined. TBF was suppressed by the presence of 0.037 pg or more of anti-TGF-β IgG. The ordinate is the response value (the strength of TBF) as defined in the section of Materials and Methods.

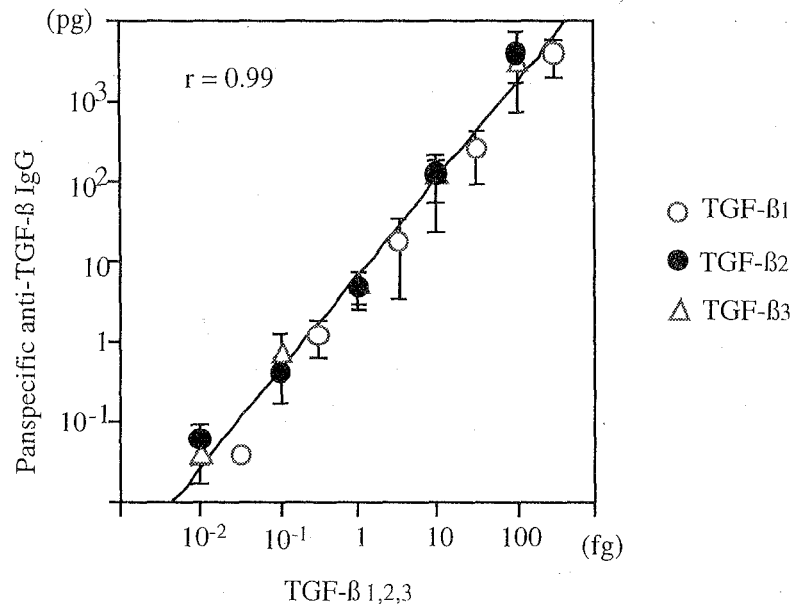


Fig. 4 The relationship between the amount of TGF-β 1, -2, and -3 and the amount of the pan-specific anti-TGF-β IgG necessary to nullify the effect of TGF-β. A linear correlation (correlation coefficient 0.99) was observed between the amount of TGF-βs (abscissa) and that of the pan-specific anti-TGF-β IgG (ordinate). The values for TGF-β1, -2, and -3 isoforms were on the same linear line, indicating that hydra did not discriminate these different forms of TGF-β. Values are means ± SEM (n=3-5).

The amount of TGF- β in CSF

We determined the amount of TGF- β in Fatg CSF from the minimum amount of anti-TGF- β IgG necessary to suppress TBF by the method described above. In Fatg CSF, the mean amount of TGF- β was 1.38 pg/ml (range 0.1-9.5 pg/ml) (Table 1). Since all TGF- β s had a similar effect on the TBF as stated above, we can not determine the amount of each individual TGF- β . No TGF- β activity was detected in Sed CSF (Table 1) because suppressed TBF in the absence of anti-TGF- β IgG.

Table 1 TGF- β content of Sed CSF (n=8) and Fatg CSF (n=8) estimated by the hydra bioassay.

Samples	TGF- β concentration (pg/ml)	Standard error	Range
Sed CSF	Not detected	-	-
Fatg CSF	1.38	1.72	0.1 -9.5

The amount of TGF- β s in Fatg CSF was determined from the linear relationship between the amount of the TGF- β and the minimum amount of the anti-TGF- β IgG necessary to suppress TBF (Fig. 3).

Discussion

We applied TBF to examine the changes in biologically active peptides in the rat CSF after exhaustive exercise. TBF was suppressed after exhaustive physical exercise. Sed CSF strongly suppressed TBF induced by GSM at all concentrations examined, while the suppressive activity of Fatg CSF was weak. Since Fatg CSF mixed with Sed CSF, showed the same effect as Fatg CSF, a substance in Fatg CSF was suggested to eliminate the suppressive effect of various substances in Sed CSF on TBF. Since the mixture of TGF- β and Sed CSF had the same effect as that of Fatg CSF, TGF- β or TGF- β -like activity may increase in CSF after exhaustive exercise. Fatg CSF treated with the anti-TGF- β IgG gave a TBF similar to that observed with Sed CSF (Fig. 2). In contrast to the effects of TGF- β s, all other members of the TGF- β superfamily that we had examined were observed to suppress the TBF (chapter I). In the same study, we also examined the effects of the latent TGF- β on the TBF, and found that only active TGF- β nullified the suppression of TBF (chapter I). It is likely that active TGF- β or TGF- β -like activity increases in CSF after exhaustive exercise.

The amount of TGF- β in Fatg CSF estimated by the hydra bioassay (1.38 ± 1.72 pg/ml) was somewhat different from that previously estimated by a standard TGF- β assay using mink

lung epithelial cells (270 ± 7.3 pg/ml) (Inoue, et al., 1999). TGF- β activity in Sed CSF was not detected by the hydra bioassay though slightly detected by the mink lung epithelial cell assay (Inoue, et al., 1999). The differences between the activity of TGF- β detected by the hydra bioassay and the mink lung epithelial cell assay could be due to the difference in the biological traits in the two systems. We have reported that the effect of TGF- β on the TBF of hydra did not differ among isoforms (Fig. 2 and chapter I). On the other hand, mink lung epithelial cells are reported to have varying sensitivity to the different isoforms of TGF- β (Cheifetz et al., 1990). The discrepancy in the estimated amount of TGF- β in the CSF may be due to different sensitivities of these biological systems to the TGF- β isoforms. The hydra assay is sensitive to many biologically active substances, so that it may be more susceptible to the assaying conditions. Despite the differences in the TGF- β values estimated by these two assay methods, both assays showed a larger amount of the TGF- β in Fatg CSF than in Sed CSF. The use of CRF in the experiment to determine the relationship between TGF- β and the anti-TGF- β IgG may also be in part responsible for the discrepancy. CSF is a complex mixture containing a lot of suppressive substances, some of which may interfere with the effect of TGF- β . We tried to use Sed CSF instead of CRF to determine the relationship between the anti-TGF- β IgG and TGF- β . However the experimental error was large because the suppressive activity of Sed CSF often changed after a few cycles of freezing and thawing.

Higher serum levels of TGF- β have been reported in patients with chronic fatigue syndrome (Chao et al., 1991), and TGF- β -like activity in the CSF of rats is elevated by exhaustive exercise (Inoue, et al., 1999). Although it is unknown whether TGF- β causes fatigue or fatigue causes an increase in TGF- β , it appears likely that TGF- β plays a critical role in fatigue.

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CHAPTER III Studies on a bioactive substance in rat cerebrospinal fluid after stimulation by an aversive quinine taste

Section 1 Identification of a bioactive substance in rat cerebrospinal fluid after stimulation by an aversive quinine taste using the hydra bioassay

Introduction

Taste plays an important role in the regulation of food and fluid intake in animals. Dopamine (Mark et al., 1994; Martel et al., 1996; Schneider, 1989), opioids (Doyle et al., 1993; Levine et al., 1985; Rideout et al., 1996), and benzodiazepines (Berridge et al., 1995; Cooper, 1982; Gray et al., 1995) are relevant substances to the palatability of foods. Morphine and benzodiazepine agonists enhance not only feeding but also the ingestive reaction by a taste reactivity test (Rideout et al., 1996, Berridge, 1995). Dopamine might be involved in incentive motivation of food reward rather than assessment of palatability (Mark, et al., 1994). Thus, neuroactive substances have been suggested to be involved in the palatable food intake. However, studies about the released substances in the brain after stimulation by an aversive taste are quite limited. Calcitonin gene-related peptide (CGRP)-like immunoreactivity levels in the gustatory insular cortex were increased significantly by strong aversive taste stimuli (Yamamoto et al., 1990). Acetylcholine has been suggested to be released in the insular gustatory cortex after the aversive taste stimuli (Shimura et al., 1995). However, acetylcholine might be mainly related to the memory formation of the taste aversion (Miranda et al., 1999; Yamamoto et al., 1998). Thus, little is known about substances released in the brain after the aversive taste stimuli, though the aversive taste information may be transmitted via a humoral factor in the brain.

1 We searched for a substance released in the brain from cerebrospinal fluid (CSF) of rats after receiving an unpleasant taste (quinine). We injected CSF obtained from rats which was stimulated by an aversive quinine solution into the oral cavity (quinine CSF) into the brain of mice to study the humoral transmission of the aversive sensation related to the quinine taste, and observed the intake of highly palatable sucrose solution in mice administered with the quinine CSF.

Then, we examined a candidate of the active substance in the quinine CSF using the Hydra behavioral test, which utilizes a tentacle ball formation (TBF), a component of the feeding response of Hydra, elicited by S-methyl-glutathione (GSM) (Hanai, 1981). TBF elicited at five different GSM concentrations showed a specific pattern of modulation for each

biologically active substance. The GSM-elicited TBF was modified by many biologically active peptides at low concentrations but not by neurotransmitters with a lower molecular weight such as catecholamines (Hanai et al., 1987; Hanai et al., 1989; Manabe et al., 2000). This system is useful to examine biologically active peptides in a small amount of biological samples (Inoue et al., 1999). In the present study, we compared the modulation of the GSM-elicited TBF by the quinine CSF with a panel of known biologically active peptides. We examined affects of a substance released in the quinine CSF in mice, and determined a candidate responsible for the effects using Hydra behavioral test.

Experiment 1

Materials and Methods

CSF sampling

Eight-week old male Wistar rats (Nihondobutsu, Osaka, Japan) were kept at 22 ± 2 °C in a humidity-controlled environment on 12h light/12h dark cycle. Food and water were available *ad libitum* until the day before the experiment. Rats were randomly divided into two groups: a quinine group and a control group. In the quinine group, rats were implanted with an intraoral cannula at least 3 days before injection of quinine solution (Shimura et al., 1995), and given quinine-HCl dissolved in distilled water (10^{-4} M) through the cannula for 20 min after fasting overnight. CSF was collected from cisterna magna (quinine CSF) in the rats under pentobarbital anesthesia 1.5 hr after the beginning of quinine infusion (Yamamoto et al., 2000). Control CSF was collected from non-treated rats after fasting overnight to avoid the influence of feeding to the hydra response (Hanai, et al., 1989) in the same manner.

CSF injection to mice

Five-week-old male Std ddY mice (Japan SLC, Hamamatsu, Japan) were housed individually in standard cages (33x 23x12 cm) under controlled conditions of temperature (22 ± 2 °C) and 12h light/12h dark cycle. Food and water were available *ad libitum* until the day before the experiment.

For training to drink sucrose solution, after deprivation of water and food for 1 hr from the beginning of the dark period, mice were given only 5 % sucrose solution (w/w) for 1 hr once a day for 3 consecutive days. On the 4th day, mice were anesthetized with pentobarbital, and surgically implanted with a guide cannula at the fourth ventricle. Coordinates were anteroposterior = - 6.0 mm from bregma, lateral to right = 0.2 mm, and dorsoventral = -4 mm (Franklin and Paxinos, 1997). After surgery, a dummy cannula was placed in the guide cannula to prevent occlusion. The sucrose intake training continued for another five days to allow recovery from surgery.

The effects of both the control CSF and quinine CSF were examined on each mouse. First, control CSF (2 μ l) was injected, and then 2 days later, quinine CSF (2 μ l) was injected using a microsyringe through the cannula over 1 min. The mice were given a 5 % sucrose solution just after CSF injection, and the intake during 30 min was determined.

All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985).

Hydra culture and behavioral test

Hydra japonica was cultured as described previously (Hanai, 1998; Hanai et al., 1995; Manabe, et al., 2000). The Hydra behavioral test was described previously (Manabe, et al., 2000). Briefly, ten hydras were incubated with a test sample in a dish containing 2 ml of PIPES solution (1 mM PIPES and 1 mM CaCl_2 , pH 6.2). Then, GSM was added at various final concentrations (0.1, 0.3, 3, 10, and 50 μ M) to stimulate the TBF. The number of hydras exhibiting TBF was counted each minute from 6 to 10 min after stimulation under a binocular microscope (8 x). The response was expressed by the following equation (Hanai and Matsuoka, 1995). Response = Sum of hydras exhibiting the TBF every minutes from 6 to 10 minutes / Total number of hydras (n=10). A response value >3.0 was judged to be no suppression of activity, while <2.4 was considered to be suppressive.

The effect of rat CSF after quinine stimuli on TBF

The CSF samples were diluted with 0.2% PRIONEX (Merck, Darmstat, Germany) in the PIPES solution until no suppressive effect was observed since TBF was suppressed by the control CSF which contained various biologically active substances. The suppressive activity of a sample was expressed as the maximum dilution at which the suppression of the TBF was still observed.

Further, an equal mixture of quinine CSF and control CSF was subjected to the Hydra behavioral test. When TBF was suppressed by the original sample, it was diluted with 0.2% PRIONEX until the effect disappeared.

Pronase treatment of CSF and molecular size fractionation

The quinine CSF (50 μ l) or the control CSF (50 μ l) was incubated with 100 μ g of pronase (Calbiochem Novabiochem Corp, La Jolla, CA) in 0.1 M PBS in a final volume of 100 μ l at 37 °C for 3 hr. Control reaction mixtures were incubated without pronase. To stop the reaction, 0.1 N HCl (80 μ l) was added. These samples (1 μ l) were subjected to the hydra assay and the TBF was examined at 3 μ M GSM. The quinine CSF was fractionated into low and high molecular weight fractions by ultrafiltration membranes, with cutoff molecular weights of 3,000, 10,000 and 30,000 (Microcon-3, 10, and 30, Millipore Corp, Bedford, MA). All size

fractions were subjected to the Hydra behavioral test.

Statistics

All statistical tests were done using StatView (SAS Institute Inc., Cary, NC). The data were analyzed by the one-way ANOVA and were followed by the *post-hoc* Tukey test. Comparison in two groups was made using the Student's *t*-test.

Results

The sucrose intake in mice injected with CSF

The sucrose intake of the mice injected with quinine CSF was significantly suppressed compared to that of mice injected with control CSF (Fig. 1). When the control CSF was used instead of the quinine CSF at the 2nd injection, the intake of the sucrose solution was not significantly different from that after the first control CSF injection. This implies that a substance in the quinine CSF suppressed the sucrose intake and that this substance also participated in the suppression of the intake by quinine. Then, we examined the quinine CSF by the Hydra behavioral test.

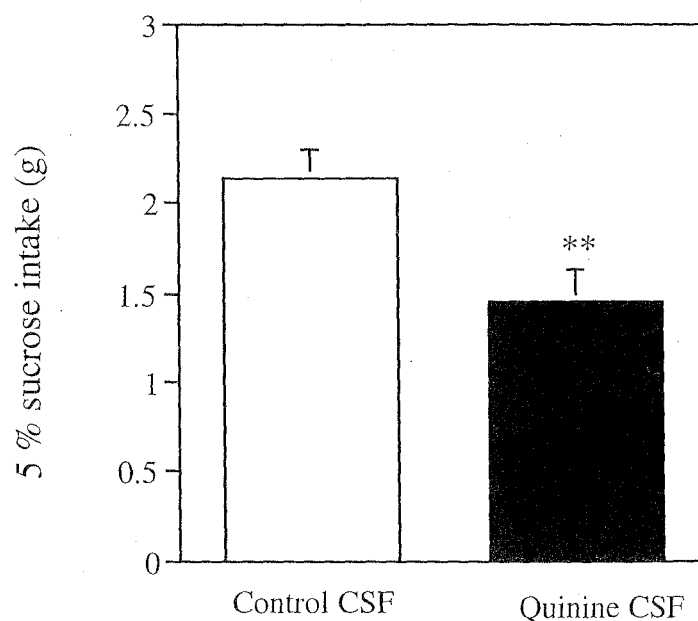


Fig. 1 The effect of injection of rat CSF on 5 % sucrose intake in mice. Control CSF or quinine CSF (2 μ l) was injected into the fourth ventricle of mice, and the sucrose intake was examined during 30 min after the injection. Quinine CSF significantly decreased the intake of sucrose solution (**; $p < 0.05$, the paired *t*-test). Values are means \pm SEM ($n=16$).

The effect of quinine CSF on TBF

The suppressive effect of the quinine CSF on TBF was weaker than that of the control CSF at 3 μ M GSM ($F(2,18)=12.146$, $p=0.0005$) (Fig. 2). We have a lot of observations that TBF have been modulated independently by many peptides (Hanai et al. 1987, 1989; Manabe et al. 2000) or monoclonal antibodies (Sakaguchi et al., 1991) at different GSM concentrations. Then, we analyzed TBF data at each GSM concentration by one way ANOVA followed by multiple comparisons. The effects of the mixture of the quinine CSF and the control CSF on TBF were similar to that of the quinine CSF (Fig. 2). These results suggested a substance that specially nullified the suppressive activity to response to GSM especially at the 3 μ M in the quinine CSF.

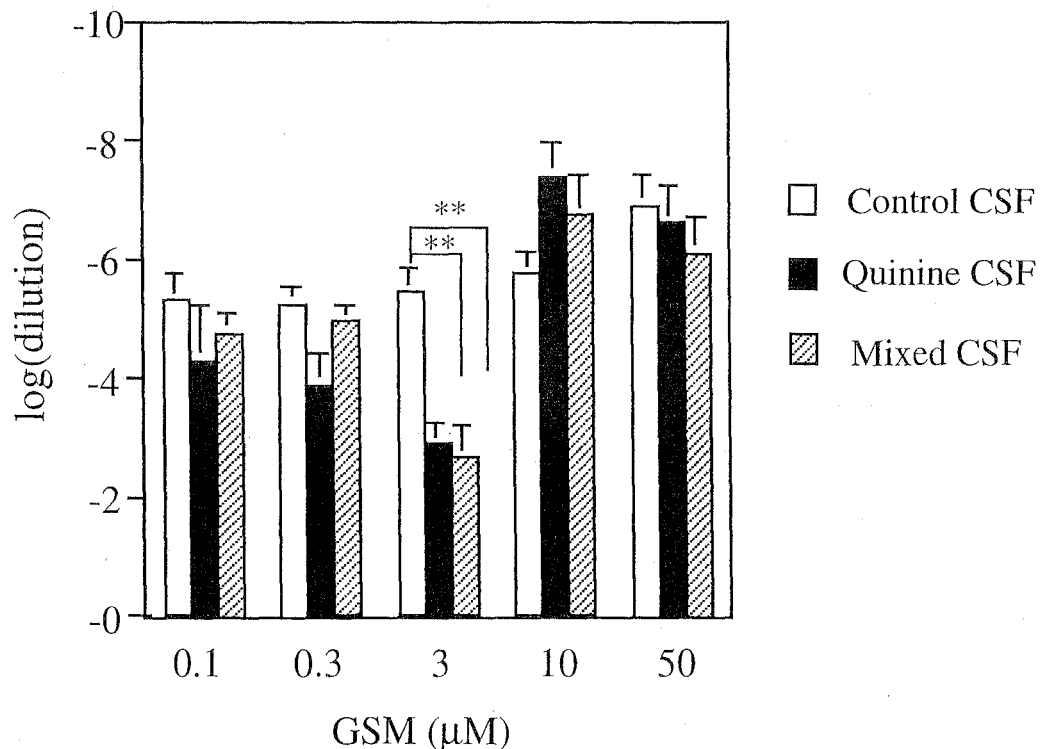


Fig. 2 Suppression of TBF by control CSF, quinine CSF, and a mixture of control CSF and quinine CSF. The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. The higher bar indicates stronger suppression of the Hydra behavioral response. Values are means \pm SEM ($n=7$). The data were analyzed by the ANOVA and the *post-hoc* Tukey test. **; $p < 0.01$.

The effect of CSF pretreated with pronase and molecular size fractionation

The nullifying effect of the quinine CSF on TBF at 3 μ M GSM disappeared by pretreatment with pronase ($F(3,16)=29.397$, $p=0.0001$) (Fig. 3), suggesting that a substance responsible for the nullifying effect in the quinine CSF is a peptide. Size-fractionation of the quinine CSF revealed the presence of the nullifying activity in the high molecular weight fraction larger than 3,000 daltons (Fig. 4 A) but lower than 30,000 daltons (Fig. 4 C). Similar amounts

of the activity were found in both fractions above and below 10,000 daltons (Fig. 4 B). Thus, the substance in the quinine CSF responsible for the nullification appeared to be a peptide of molecular weight about 10,000 daltons.

We had examined the effect of a number of peptides including CGRP on TBF (Hanai et al., 1989, Chapter I), but no peptide showed the same effect to be considered as a candidate. Therefore, in the next study, we focused on possible contribution of benzodiazepine receptor and its endogenous ligand to the effects of the substance in the quinine CSF since benzodiazepines are involved in the palatability of foods (Berridge and Pecina, 1995; Cooper, 1982; Gray and Cooper, 1995).

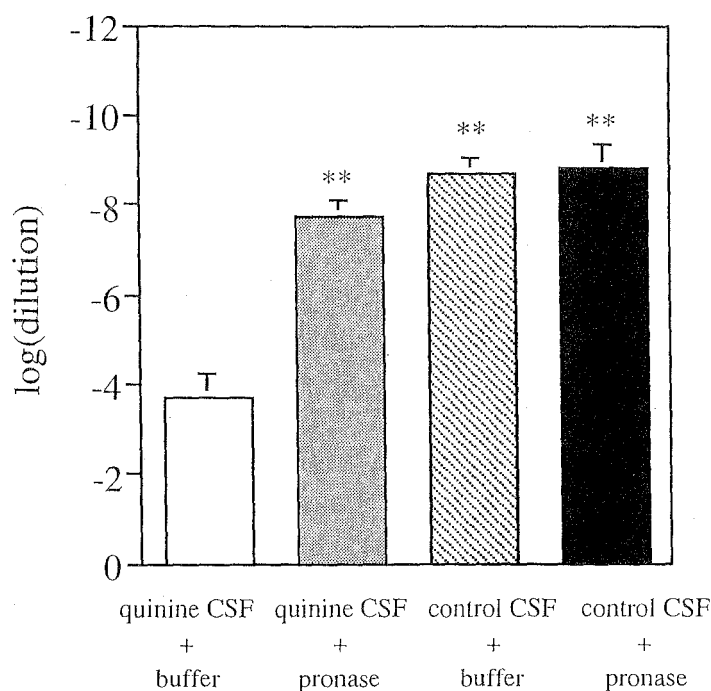


Fig. 3 Suppression of TBF response after treatment with pronase. After treatment of quinine CSF with pronase, the suppressive activity was increased, whereas the treatment of control CSF did not change the suppressive activity. Suppression of the TBF response was examined after stimulation with 3 μ M GSM. The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. 0.1 M PBS was used as the buffer. Values are means \pm SEM (n=5). The data were analyzed by the ANOVA and the *post-hoc* Tukey test. **: $p < 0.01$.

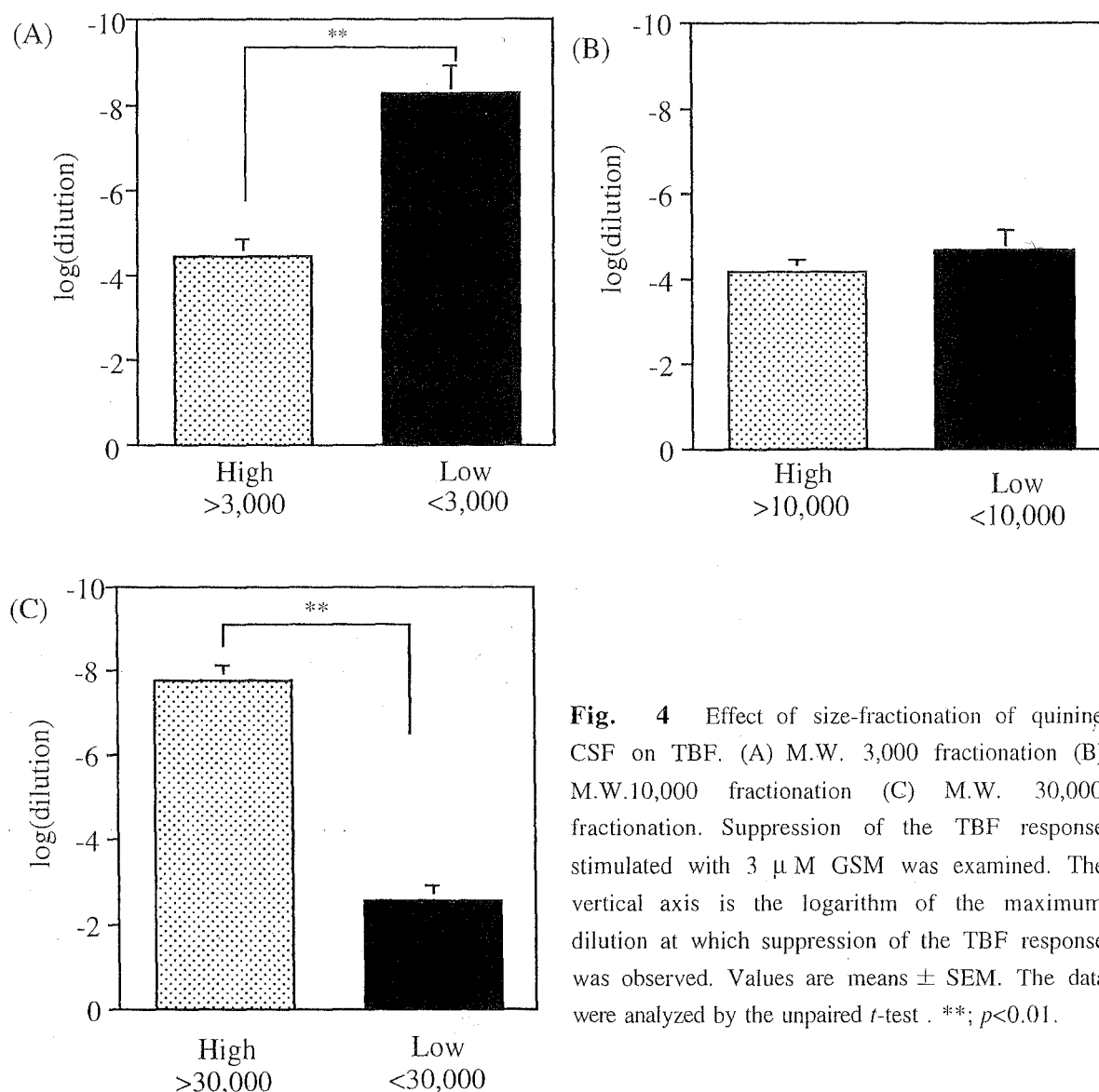


Fig. 4 Effect of size-fractionation of quinine CSF on TBF. (A) M.W. 3,000 fractionation (B) M.W. 10,000 fractionation (C) M.W. 30,000 fractionation. Suppression of the TBF response stimulated with 3 μM GSM was examined. The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. Values are means \pm SEM. The data were analyzed by the unpaired *t*-test. **: $p < 0.01$.

Experiment 2

Benzodiazepines are reported to be related to the increase in the intake of palatable foods (Berridge and Pecina, 1995; Cooper, 1982; Gray and Cooper, 1995), although their effects are only exogenous. The benzodiazepine receptor is widely distributed in the brain, while an endogenous benzodiazepine-like ligand is unknown. Diazepam binding inhibitor (DBI) is the only known endogenous ligand to this receptor, which is a 10-kilodalton neuropeptide present in the brain of rats and humans (Guidotti, et al., 1983; Shoyab et al., 1986). It binds to the benzodiazepine binding site on the GABA_A receptor complex (Bormann, 1991; Guidotti, 1991), and acts as an inverse agonist, which elicits effects opposite to those of benzodiazepines (Costa, 1991; Ferrero et al., 1986; Guidotti et al., 1983). Injection of DBI into the brain reportedly

produced anxiogenic effects in the conflict test (Guidotti, 1991). Further, increases in DBI-like immunoreactive compounds have been reported in depressive patients (Ferrero et al., 1988; Roy, 1991) and in alcohol-dependent rats (Adinoff et al., 1996; Katsura et al., 1998). Katsura et al. (1995) also reported that expression of DBI mRNA was elevated in the brains of ethanol-withdrawn mice after its chronic treatment. Taken together, DBI might be related to an aversive sensation as a biological active molecule. Since injection of benzodiazepine agonists into the brain acts to enhance palatability, a benzodiazepine inverse agonist DBI may be involved in the aversive feeling as to taste. Therefore, we examined the affects of DBI on TBF and possibility as a candidate for the peptide in the experiment 1.

Materials and Methods

Methods for the CSF sampling and injection to mice were presented in the experiment 1. Hydra culture and behavioral test were also described in the experiment 1.

The effect of DBI on TBF

We examined the effect of a DBI peptide fragment (Bachem AG, Bubendorf, Switzerland) on TBF in the presence of both the DBI fragment (1 fg) and 1 μ l of the control CSF. The DBI peptide fragment is composed of 20 amino acid residues from Gln 51 to Lys 70 of the human DBI sequence, which has been reported to be biologically active (Dong et al., 1999). The stock solution of the peptide was prepared with distilled water, and added to the TBF assay system after dilution with 0.2% PRIONEX. The control CSF, which contained various biologically active peptides, strongly suppressed the TBF induced by GSM at all concentrations examined (Hanai, et al., 1989; Inoue, et al., 1999). The sample was diluted with 0.2% PRIONEX until the suppressive effect disappeared.

Effect of benzodiazepine receptor preparation

Benzodiazepine receptor preparation (Research Biochemicals, Inc. (RBI), Natick, MA) was dissolved at a concentration of 50 mg/ml in 0.25 M NaH_2PO_4 buffer (pH 7.4) (Imaizumi et al., 1994). The quinine CSF (5 μ l) or the control CSF (5 μ l) and the receptor preparation (20 μ l) were mixed and incubated for 1 hr on ice. Control reaction mixtures were incubated without receptor preparation. The mixture was filtrated through an ultrafiltration membrane (Microcon-30), and subjected to the TBF assay at 3 μ M GSM. The sample was diluted with 0.2% PRIONEX until the suppressive effect disappeared.

N-Methyl- β -carboline-3-carboximide (FG7142) (RBI), which was bound to the benzodiazepine receptor (Cooper, 1986; Cooper et al., 1988), was used to examine the specificity of the binding of the substance in the quinine CSF to the receptor preparation. FG7142 solution (2 mg/ml, 10 μ l) and benzodiazepine receptor preparation (20 μ l) were mixed

and incubated for 15 min on ice. Then, the quinine CSF (5 μ l) was added and incubated for another 30 min on ice. The resultant mixture (1 μ l) was subjected to the Hydra behavioral test and observed at 3 μ M GSM. The sample was diluted with 0.2% PRIONEX until the suppressive effect disappeared.

Flumazenil pretreatment

The selective benzodiazepine receptor antagonist, flumazenil (a gift from Yamanouchi Pharmaceutical Co. Ltd, Tokyo, Japan), was suspended in physiological saline containing 0.5 % carboxymethyl cellulose (CMC saline). Flumazenil (20 mg/kg) was administered intraperitoneally (i.p.) 20 min before CSF injection and CMC saline was used as control. Mice were treated and trained as above (experiment 1). On the first day for injection, CMC saline was administered i.p., 20 min before the control CSF (2 μ l) injection through the cannula. The sucrose intake was determined at 30 min after the injection of CSF, and then mice were divided into two groups, CMC saline-quinine CSF group and flumazenil-quinine CSF group. Two days after the first injection, CMC saline or flumazenil was administered i.p. 20 min before quinine CSF (2 μ l) injection into the fourth ventricle through the cannula and the 5 % sucrose intake was determined.

Statistics

The statistical analysis was done in the same manner as described in the experiment 1.

Results

The effect of DBI on the TBF

The DBI fragment itself did not suppress TBF (data not shown). To examine the activity of DBI to nullify the suppressive effect of quinine CSF, we examined the activity of the mixture of DBI fragment and control CSF. The suppression of TBF at 3 μ M GSM by CSF was greatly reduced by the mixture of DBI and control CSF (Fig. 5), which was similar to that by quinine CSF.

Effect of the treatment of the quinine CSF with benzodiazepine receptor preparation

CSF was incubated with the benzodiazepine receptor preparation, which bound DBI. The mixture of the quinine CSF and the receptor preparation suppressed TBF at 3 μ M GSM to a similar extent as the control CSF ($F(3,20)=11.033$, $p=0.0002$) (Fig. 6), indicating disappearance of the nullifying effect in the original quinine CSF. When the benzodiazepine receptor preparation was preincubated with FG7142, the nullifying effect did not disappear ($F(2,12)=12.118$, $p=0.013$) (Fig. 7). These results indicate that the nullifying activity in the

quinine CSF was removed by the specific binding of the active substance to the benzodiazepine receptor in a manner similar to that of DBI.

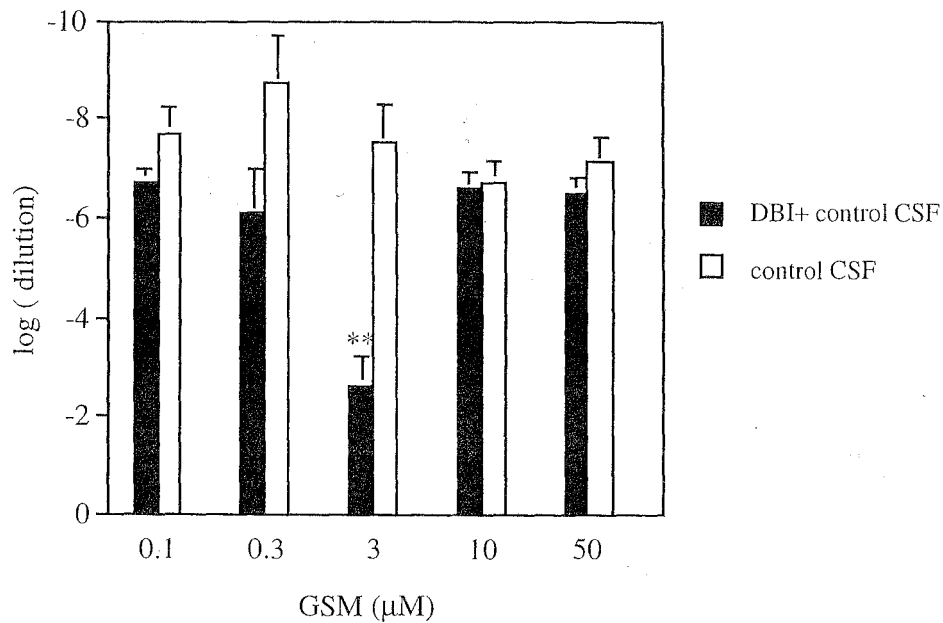


Fig. 5 Suppression of GSM response by control CSF and by a mixture of control CSF with DBI (1 fg). The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. Values are means \pm SEM ($n=5$). The data were analyzed by the unpaired *t*-test **; $p<0.01$.

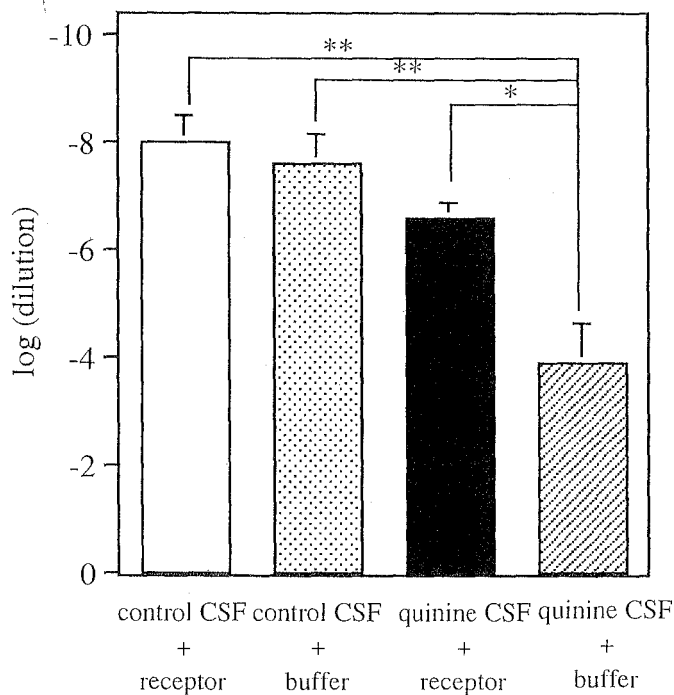


Fig. 6 Suppression of TBF response after treatment with benzodiazepine receptor preparation. Treatment of quinine-CSF with benzodiazepine receptor preparation increased the suppressive activity, whereas the same treatment of control CSF did not change the suppressive activity. Suppression of the TBF response was examined after stimulation with 3 μ M GSM. The vertical axis is the maximum dilution. Values are means \pm SEM ($n=6$). The data were analyzed by the ANOVA and the *post-hoc* Tukey test. *, $p<0.05$; **, $p<0.01$.

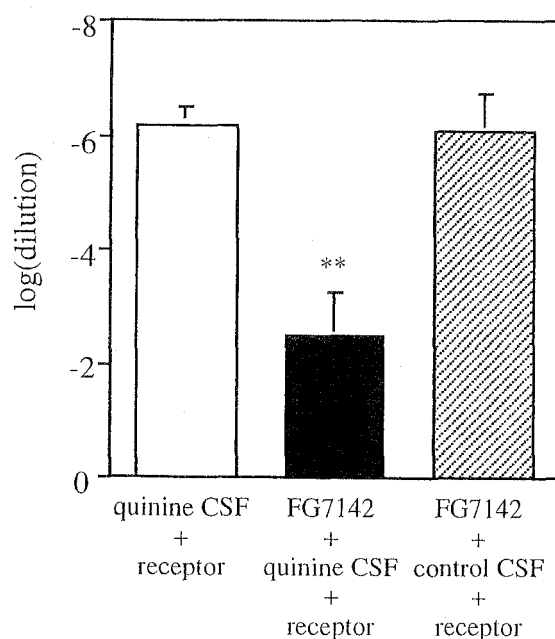


Fig. 7 Effect of the mixture of quinine CSF with benzodiazepine receptor preparation after pretreatment with FG7142 on TBF response. After pretreatment of quinine CSF with FG7142, the nullifying effect of benzodiazepine receptor preparation on quinine CSF activity disappeared. FG7142 did not change the suppressive activity of the mixture of control CSF and the receptor preparation. Suppression of the TBF response was examined after stimulation with 3 μ M GSM. The vertical axis is the maximum dilution. Values are means \pm SEM (n=5). The data were analyzed by the ANOVA and the *post-hoc* Tukey test. **; $p < 0.01$.

Antagonism by flumazenil on the effect of the quinine CSF on sucrose intake

We confirmed the suppressive activity of the quinine CSF on the 5 % sucrose intake in mice pretreated with CMC saline during 30 min after the injection ($F(2,41)=4.786$, $p=0.01368$) (Fig. 8). Pretreatment with flumazenil, a benzodiazepine receptor antagonist, reduced the suppressive effect of quinine CSF on the sucrose intake and its statistical significance was disappeared (Fig. 8).

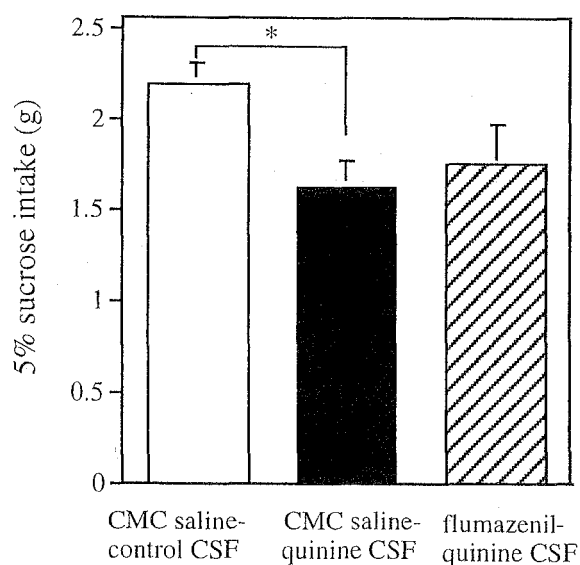


Fig. 8 The effect of pretreatment with flumazenil (20 mg/kg, i.p.) on the intake of 5 % sucrose solution. Flumazenil or CMC saline was given 20 min before injection of CSF. The sucrose intake during 30 min after the CSF injection was examined. Data are means \pm SEM (CMC saline-control CSF; n=22, CMC saline-quinine CSF; n=11, flumazenil-quinine CSF; n=11). The data were analyzed by the ANOVA and the *post-hoc* Tukey test. *; $p < 0.05$.

Discussion

The sucrose intake was significantly suppressed in mice injected with the quinine CSF, indicating the presence of the active substance in quinine CSF. The Hydra behavioral test detected a change in suppression of TBF by CSF that was accompanied with aversive quinine taste stimuli. The control CSF was suppressive at all concentrations of GSM, while the quinine CSF was not as suppressive at 3 μ M GSM. TBF was not suppressed by the mixture of quinine CSF and control CSF, suggesting that a substance in the quinine CSF nullified the suppression of TBF caused by various substances in the control CSF at 3 μ M GSM. By means of pronase treatment and size-fractionation of quinine CSF, the nullifying effect of suppressive activity in the quinine CSF disappeared, suggesting that a proteinous substance of about M.W.10,000 was responsible for this effect. We have examined the effect of a number of peptides on TBF. No peptides examined previously other than the DBI fragment nullified selectively the suppression of the response at 3 μ M GSM (Hanai, et al., 1989; Manabe, et al., 2000).

In this study, we focused on DBI. DBI was reported as an inverse agonist to the benzodiazepine receptor, which elicits effects opposite to those of benzodiazepines (Costa, 1991; Ferrero et al., 1986; Guidotti et al., 1983). DBI is the only endogenous ligand to the benzodiazepine receptor. No relationship between DBI and feeding has been reported at present, while benzodiazepines are reported to be related to the palatability (Berridge and Pecina, 1995; Cooper, 1982; Gray and Cooper, 1995). From the results in the experiment 2, the nullifying effect on the TBF at 3 μ M GSM in the Hydra behavioral test is believed to be mediated principally by an increase in the DBI-like activity in the CSF. The effect of the mixture of the DBI peptide fragment and the control CSF on TBF in the present study resembled that of the quinine CSF. The quinine CSF treated with the benzodiazepine receptor preparation gave TBF similar to that of the control CSF, indicating the presence of DBI-like activity in the quinine CSF. Further, the suppression of the sucrose intake by quinine CSF was reduced by the pretreatment with flumazenil. It suggested the existence of a substance of whose activity is mediated via benzodiazepine receptors. These results suggest that a DBI-like peptide is released by the aversive quinine stimuli, and it acts via benzodiazepine receptors.

Flumazenil did not completely antagonize the suppressive effects of quinine CSF on the sucrose intake. It is likely that other factors besides DBI participate in the suppressive effect on the sucrose intake of the quinine CSF. Also, the suppression of the sucrose intake by the DBI fragment alone requires a high dose when it was injected into the mouse brain (unpublished observation).

In this study, DBI-like activity increases in CSF after the aversive quinine taste stimuli. The meaning of the increase in DBI by quinine intake is not clear but higher CSF levels of DBI may be related to the feeling of disgust after intake of unfavorable foods. An increase in DBI in the quinine CSF, we reported here, might explain why the intake of unfavorable foods induces

the feeling of disgust. Further studies are necessary to determine if DBI is specific to the feeling of disgust as to feeding.

In conclusion, we suggested that aversive sensation after the quinine stimulation might be transmitted via a peptide in the experiment 1 and it was a DBI-like molecule which binds benzodiazepine receptors in the experiment 2.

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Section 2 Effect of diazepam binding inhibitor on the fluid intake and preference in mice

Introduction

In section 1, we suggested that DBI in the CSF from rats received by an aversive quinine-HCl increased using Hydra bioassay. DBI is the only endogenous ligand for benzodiazepine receptor. Benzodiazepines are reported to be related to the increase in the intake of palatable foods (Berridge et al., 1995; Cooper, 1982; Gray et al., 1995). The benzodiazepine receptor is widely distributed in the brain, while an endogenous benzodiazepine-like ligand is unknown besides DBI. DBI is categorized into inverse agonist, which has against effect to benzodiazepine (Costa, 1991; Ferrero et al., 1986; Guidotti et al., 1983). All consider these facts, we studied the involvement of DBI in the aversion of taste.

Materials and Methods

Animals

Five-week-old male Std ddY mice (Japan SLC, Hamamatsu, Japan) were housed individually in standard cages (33 x 23 x 12 cm) under controlled conditions of temperature (22 ± 2 °C) and 12h light/12h dark cycle. Food and water were available ad libitum until the day before the experiment.

Surgery

Mice were anesthetized with pentobarbital, and surgically implanted with a guide cannula at the fourth ventricle. Coordinates were anteroposterior = - 6.0 mm from bregma, lateral to right = 0.2 mm, and dorsoventral = - 4.0 mm (Paxinos, 1997). After surgery, a dummy cannula was placed in the guide cannula to prevent occlusion. At the end of the experiment, correct guide cannula placement was verified by injecting 2 μ l of thionine blue dye, followed by decapitation and dissection of the brain.

All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985).

Drugs

DBI peptide fragment (Bachem AG, Bubendorf, Switzerland) was dissolved in artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl_2 , 1 mM CaCl_2 , 1.2 mM Na_2HPO_4 , 0.27 mM NaHPO_4). aCSF was used as the control. The selective benzodiazepine receptor antagonist, flumazenil (a gift from Yamanouchi Pharmaceutical Co.

Ltd., Tokyo, Japan), was suspended in physiological saline containing 0.5 % carboxymethyl cellulose (CMC saline).

The effect of DBI peptide fragment on the intake of solution

We determined the effect of injection of DBI on 5% sucrose intake. For training to drink sucrose solution, after deprivation of water and food for 1 hr from the beginning of the dark period, mice were given only 5 % sucrose solution (w/w) for 1 hr once a day for 3 consecutive days. On the 4th day, guide cannula were implanted into the fourth ventricle. After surgery, the training for sucrose intake continued for another five days to allow recovery.

On the first day for injection, aCSF (2 μ l) was injected, and then 2 days later, DBI (10 μ g/2 μ l) was injected using a microsyringe through the cannula over 1 min. The mice were given 5% sucrose just after injection, and the intake was examined at 15, 30 and 60 min.

Furthermore, we determined the effect of DBI on the intake of water and 0.9 mM quinine HCl. For training to drink water or 0.9 mM quinine HCl, mice were water-deprived for 20 hr and then given 1 h access to the water or 0.9 mM quinine HCl once a day for 3 consecutive days. On the 4th day, guide cannula were implanted into the fourth ventricle. After surgery, the fluid intake training continued for another five days to allow recovery. The injection of DBI or aCSF was the same as above. Then, the water or 0.9 mM quinine-HCl intake was measured at 15, 30 and 60 min after injection.

In a flumazenil pretreatment experiment, on the first day for injection, CMC saline was administered i.p., 20 min before aCSF (2 μ l) injection through the cannula. The sucrose intake was determined at 15, 30 and 60 min after the injection of aCSF, and then mice were divided into two groups without bias in the intake on the first injection, CMC saline-DBI group and flumazenil-DBI group. Two days after the first injection, CMC saline or flumazenil was administered i.p. 20 min before the injection of DBI (10 μ g/2 μ l) into the fourth ventricle through the cannula and the 5 % sucrose intake was determined.

Two bottle choice test

Mice were given water and 0.05 % saccharin (Sigma Chemical Co., St Louis, USA) for 30 min after the 20 hr deprivation of water at the same time to train selective intake of the favorable fluid in two-bottle choice test. Mice were deprived of food during the two bottle choice test. After training for 7 days, mice were implanted the cannula in the fourth ventricle. After surgery, the two bottle choice test continued for another four days to allow recovery. On the first day for injection, aCSF (2 μ l) was injected, and then 2 days later, DBI (10 μ g/2 μ l) was injected into the fourth ventricle. The mice were given two-bottle just after injection, and the intake was examined at 30 min. Preference was expressed as the intake of 0.05 % saccharin divided by the total intake.

We studied the antagonism by flumazenil. After training of the two bottle choice test, mice were injected CMC saline (i.p.), 20 min before aCSF injection (i.c.v.). The preference was determined at 15, 30 and 60 min after the injection of aCSF, and then mice were divided into two groups, CMC saline-DBI group and flumazenil-DBI group, without bias in the intake on the first injection. Two days after the first injection, CMC saline or flumazenil was administered i.p. 20 min before DBI injection into the fourth ventricle through the cannula and the intake was determined.

Dose-dependent effect of DBI on the intake of sucrose

We determined the dose-dependent effect of DBI on the intake of 5 % sucrose. Mice were treated and trained the same as above mentioned way (The effect of DBI peptide fragment on the intake of solution section). DBI was prepared at 0.1, 0.3, 1.0, 3.0 $\mu\text{g}/2\ \mu\text{l}$ aCSF, and two microliter of DBI injected into the fourth ventricle of mice. The mice were given 5 % sucrose after injection, and the intake was examined at 30 min.

Statistics

All statistical tests were done using StatView (SAS Institute Inc., Cary, NC). The data were analyzed by the one-way ANOVA and were followed by the *post-hoc* Tukey test or Dunnet test. Comparison in two groups was made using the Student's *t*-test.

Results

The effect of DBI peptide fragment on the intake of solution

We confirmed that the effect of DBI on the intake of various kind of fluids. The injection of DBI (10 μg) significantly decreased the intake of 5 % sucrose at 15, 30 and 60 min after injection of DBI (Fig. 1a). Water and 0.9 mM quinine-HCl were also suppressed by DBI at 30 and 60 min after the injection (Fig. 1b, c).

The effect of pretreatment of flumazenil on the intake of sucrose

Pretreatment of flumazenil to mice partly antagonized the suppressive effect of DBI on the intake of 5% sucrose, while the intake on the group of saline-DBI was significantly suppressed (Fig. 2). However, there was no significant difference between the group of saline-DBI and the group of flumazenil-DBI, which suggests the suppressive effect of DBI on the intake of 5 % sucrose partially antagonized by flumazenil.

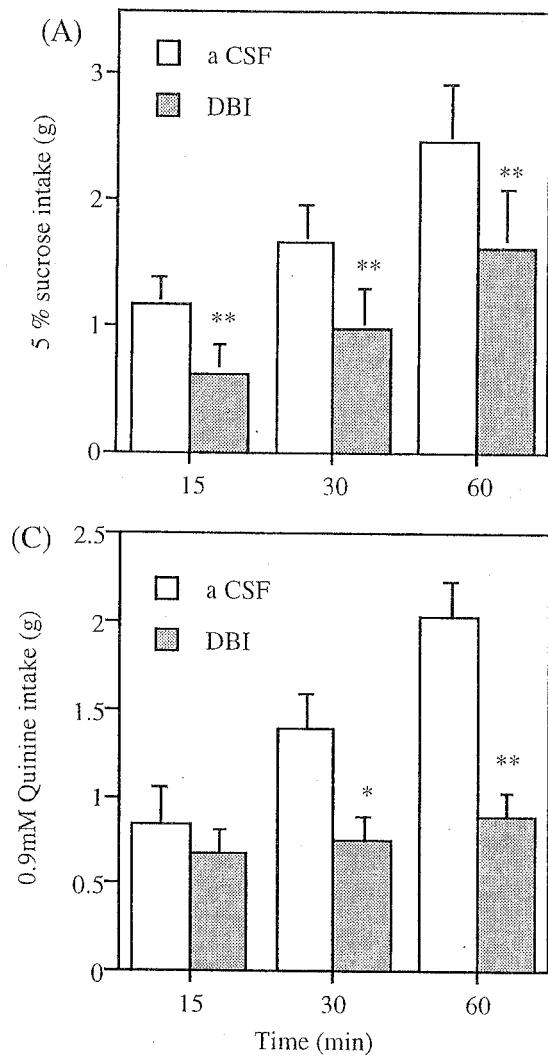


Fig. 1 The effect of injection of DBI (10 µg/2 µl) on the intake in mice. DBI was injected into the fourth ventricle of mice, and the intake was examined at 15, 30 and 60 min after the injection. (A) the intake of 5% sucrose (n=8). (B) the intake of water (n=7). (C) the intake of 0.9 mM quinine-HCl (n=8). DBI significantly decreased the intake of any solution (*; $p < 0.05$, **; $p < 0.01$, the paired t -test). Values are means \pm SEM.

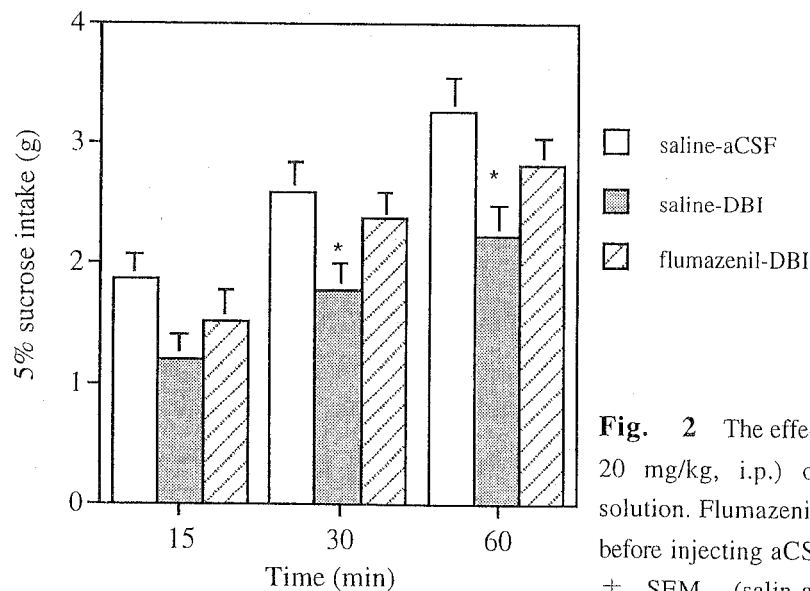


Fig. 2 The effect of pretreatment with flumazenil (20 mg/kg, i.p.) on consumption of 5 % sucrose solution. Flumazenil or CMC-saline was given 20 min before injecting aCSF or DBI (10 µg). Data were means \pm SEM (salin-aCSF; n=17, saline-DBI; n=9, flumazenil-DBI; n=8). *; $p < 0.05$, Significant different from control (Tukey test).

Two bottle choice test

Two bottle choice test was studied at 30 min after injection of DBI or aCSF, because the effect of DBI and antagonism of flumazenil is clear at 30min. The injection of DBI decreased the preference of saccharin at 30 min after injection (Fig.3). Flumazenil were antagonized the suppressive effect of preference by DBI (Fig.4). The preference of the group of CMC saline-DBI was significantly decreased compared with the group of saline-aCSF and that of flumazenil-DBI.

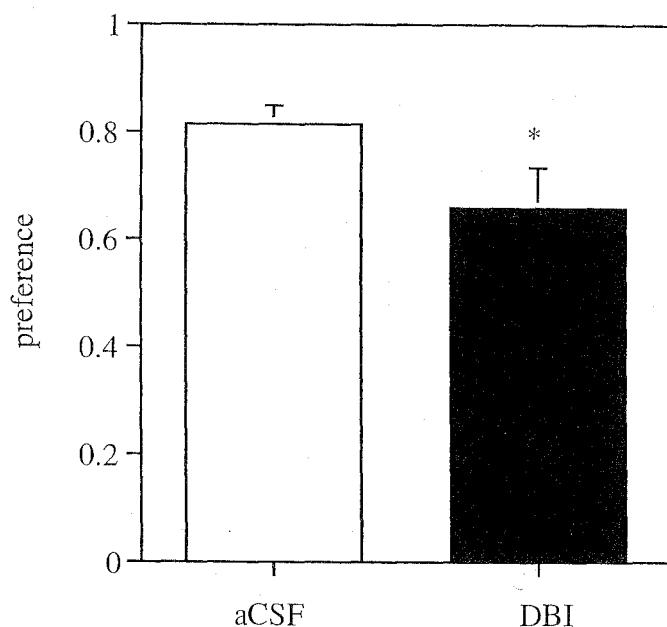
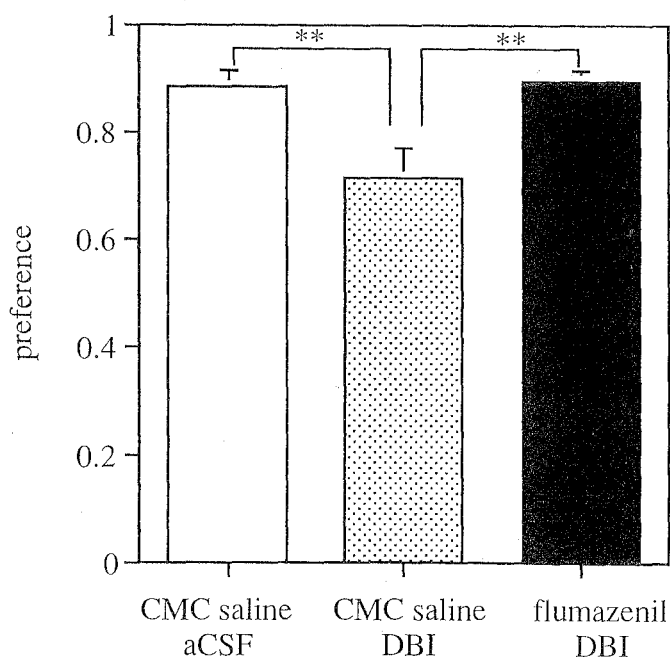


Fig. 3 The effect of DBI on the preference for 0.05 % saccharin for 30 min in the two bottle choice test. ACSF or DBI (10 μ) was injected into fourth ventricle in mice. Data are means \pm SEM (n=8). Significant difference was observed between aCSF and DBI by the paired *t*-test (*; $p < 0.05$)

Fig. 4 The effect of pretreatment with flumazenil (20 mg/kg, i.p.) on the preference for 0.05 % saccharin for 30 min in the two bottle choice test. Flumazenil and CMC-saline was given 20 min before injection (i.c.v.) of aCSF or DBI (10 μ g) in mice. Data are means \pm SEM (CMC saline-aCSF; n=10, CMC saline-DBI; n=5, flumazenil-DBI; n=5). Significant differences were observed between preference of CMC saline-aCSF and between CMC saline-DBI and CMC saline-DBI and flumazenil (**, $p < 0.01$).



Dose-dependent effect of DBI on the intake of sucrose

The dose of DBI in excess of 3 μg suppressed the intake of sucrose significantly (Fig. 5). However, the degree of suppression of sucrose was nearly identical among three dose (3, 10 and 30 μM).

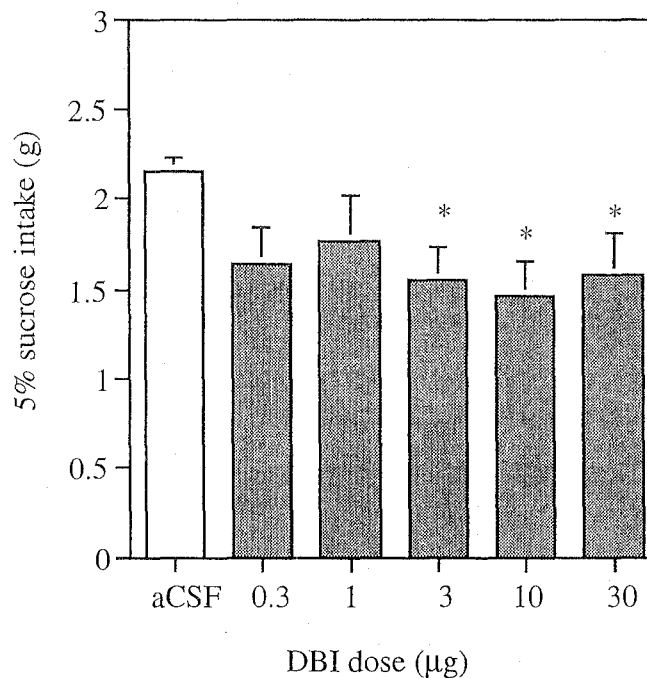


Fig. 5 The effect of DBI (0.3-30 μg) on the intake of 5% sucrose in mice. The intake of 5% sucrose was significantly decreased in excess of 3 μg DBI. Data are means \pm SEM (n; aCSF=61, 0.3 μg DBI=12, 1 μg DBI=13, 3 μg DBI=15, 10 μg DBI=9, 30 μg DBI=12, *, $p < 0.05$, Dunnett test).

Discussion

The experiment in section 1 suggested that injection of CSF from rats received quinine-HCl into the brain decreased significantly the intake of 5% sucrose in mice. This suppressive effect of the intake supposed to come from DBI in CSF from rats received by an aversive quinine-HCl. These results in here shows the effect of DBI peptide fragment into the brain on the intake in mice. The injection of DBI into the fourth ventricle decreased not only the intake of sucrose but also the intake of water and 0.9 mM quinine-HCl solution. Furthermore, DBI decreased the preference for saccharin. The suppression of the fluid intake and preference by DBI was antagonized by preinjection of flumazenil. These antagonism suggests DBI acted through benzodiazepine receptor. Furthermore, we studied DBI about the effect of dose dependent on the intake of 5% sucrose. We estimated the lower limit of the effect of DBI on the suppression of the intake was 3 μg (Fig. 5).

It is not clear how the antagonism by flumazenil was perfect in the Fig. 2, while

flumazenil perfectly antagonized the suppressive effect of preference for saccharin in the Fig.4. It was reported that flumazenil itself might act as the inverse agonist (De Vry et al., 1985). On the other hand, flumazenil was reported to act as the agonist (Haefely et al., 1988). Therefore, we examined the effect of flumazenil itself on the intake of 5 % sucrose, which resulted in tending to decrease the intake, while that effect was not significant (data not shown). The discrepancy of effect by flumazenil between Fig.2 and Fig.4 might come from individual difference.

We need more research to clarify whether the effect of DBI on the intake is dependent on the kind of fluid. DBI suppresses even the intake of water, which is not related to any tastes, suggests DBI has the suppressive effect to all fluid. Interestingly, the suppressive effect of DBI on the intake of quinine-HCl as a bitter taste and sucrose as a sweet taste is stronger than that on the intake of water. In other words, DBI seems to suppress the intake of palatable fluid and unpalatable food more than water. These results suggest the DBI affects palatability of taste in mice.

The suppression by DBI on the intake of 5 % sucrose will be the most important suggestion in the sensation of the taste. At present, injection of DBI into the brain reportedly produced anxiogenic effects in the conflict test. Furthermore, increases in DBI-like immunoreactive compounds have been reported in depressive patients (Ferrero et al., 1988; Roy, 1991) and in alcohol-dependent rats (Adinoff et al., 1996; Katsura et al., 1998). Katsura et al. (1995) also reported that expression of DBI mRNA was elevated in the brains of ethanol-withdrawn mice after its chronic treatment (Katsura et al., 1995). However, there is no research about relationship between taste and DBI. Taken together, DBI might be related to an aversive sensation as a biological active molecule after the stimulation of an aversive taste. Since injection of benzodiazepine agonists into the brain acts to enhance palatability (Berridge and Pecina, 1995; Cooper, 1982; Gray and Cooper, 1995; Yamamoto et al., 1998), a benzodiazepine inverse agonist DBI may be involved in the aversive feeling as to taste.

In this study, we provide evidence for a novel substance in the brain as to quinine aversive taste. To determine the distinct relationship between DBI and taste, we will need additional study such as the effect of DBI on the behavior of mice and determination the content of DBI in CSF.

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SUMMARY

This research was about released substances in the brain related to physiological change using hydra bioassay system. The finding in each chapter are summarized as follows:

Chapter I

Since there is a little amount of bioactive substance in the cerebrospinal fluid (CSF), it was tough to find the change of substances in the CSF related to physiological change. We applied the hydra bioassay, which is useful to screen active peptides in small amounts of biological samples containing very low concentrations of peptides. At first, we constructed the hydra bioassay system appropriate to find the substances in CSF. The tentacle ball formation (TBF) of Hydra elicited by S-methylglutathione (GSM) was modulated by a number of biologically active peptides. Hydra fed with *Artemia*, which had been hatched in common salt solution supplemented with LiCl and ZnCl₂, effectively developed the TBF by trypsin treatment. After Hydra were treated with 100 pg/ml trypsin for 10 min, TBF was sensitively suppressed by acidic fibroblast growth factor and other biologically active peptides for longer than 10 hr. Different peptides except for transforming growth factor beta (TGF- β) gave different suppressive patterns in a specific way depending on individual peptides. However, TGF- β was unique in that it did not suppress the TBF, but it eliminated suppression of the TBF caused by other peptides. Only active TGF- β eliminated the suppression of the TBF by other peptides. The latent form of TGF- β was not suppressive nor showed the elimination. Other closely related peptides examined, members of TGF- β family, were all-suppressive for the TBF. These results indicate that all peptides examined but TGF- β suppressed the TBF in a specific way depending on individual peptides. This assay system would be useful to learn about the type of biologically active substances in a biological sample by observing the modulating activity of the biological sample.

Chapter II

We have proposed the participation of transforming growth factor beta (TGF- β) in fatigue from a study using TBF of hydra as a bioassay [K. Inoue, H. Yamazaki, Y. Manabe, C. Fukuda and T. Fushiki, Transforming growth factor-beta activated during exercise in brain suppresses spontaneous motor activity of animals. Relevance to central fatigue, Brain Res. 64 (1999) 145-53]. The suppression of the CSF obtained from rats after exhaustive exercise (Fatg CSF) was marked lower than that of sedentary rats (Sed CSF). Addition of transforming growth factor-beta (TGF- β), which is the only substance known to nullify TBF, to CSF of the sedentary rat reproduced this change in the suppression of the TBF. The different effect between Sed CSF and Fatg CSF on TBF was nullified by the treatment of Fatg CSF with

anti-TGF- β IgG. This suggested that TGF- β in Fatg CSF participates in the GSM-induced TBF. TGF- β overcomes the TBF-suppressing effect of corticotropin-releasing factor (CRF) added to the TGF- β -containing solution. A linear relationship was observed between the minimum amount of anti-TGF- β IgG necessary to nullify the TBF-suppressing effect of CRF in a wide range of TGF- β concentrations. This amount of anti-TGF- β IgG corresponds to the amount necessary to nullify the effect of TGF- β in the solution. By using this relationship, we quantitatively examined the TGF- β like activity in the Fatg CSF, using the anti-TGF- β IgG and the hydra bioassay. No TGF- β activity was detected in the Sed CSF, whereas the amount of TGF- β in the Fatg CSF was 1.38 pg/ml. These results also confirm that fatigue is related to an increase in TGF- β .

Chapter III

Section 1

CSF from rats after stimulation by aversive quinine taste (quinine CSF) administered into the fourth ventricle of mice suppressed their intake of 5 % sucrose solution. We examined the effects of CSF on the TBF of Hydra to determine the change in CSF components associated with the aversive taste stimuli. The suppressive activity of the quinine CSF on TBF at 3 μ M GSM was markedly lower than the CSF obtained from the control rats (control CSF). The pronase-treated quinine CSF had suppressive activity similar to that of the control CSF. The active principle passed through an ultrafiltration membrane, with a cutoff molecular weight of 30,000 daltons, but not 3,000 daltons. A peptide fragment of the diazepam binding inhibitor (DBI) nullified the suppression of TBF at 3 μ M GSM by control. The nullifying activity of the quinine CSF was not observed after treatment with the benzodiazepine receptor preparation that was able to bind DBI. After application of flumazenil, a benzodiazepine receptor antagonist, to mice the suppressed intake of 5 % sucrose solution by the quinine CSF partially recovered. In conclusion, the quinine CSF was suggested to contain a DBI-like substance.

Section 2

To determine the effect of DBI on the fluid intake, we injected DBI fragment into the fourth ventricle in mice. DBI suppressed the intake of 5% sucrose, water and 0.9mM quinine-HCl and the preference for 0.05% saccharin. Administration (i.p.) of flumazenil, benzodiazepine receptor antagonist, 20 min before the injection of DBI (i.c.v.) antagonized the suppressive effect of DBI on the intake and the preference for saccharin. We also studied the dose dependent effect of DBI on the intake of 5% sucrose. Injection of DBI in excess of 3 μ g suppressed the intake of 5% sucrose in mice. These results suggest that DBI has a suppressive effect of fluid intake through the benzodiazepine receptor in mice.

ACKNOWLEDGEMENTS

The author is sincerely grateful to Dr. Tohru Fushiki, Professor of Nutrition Chemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, for useful advice, helpful discussions and his continuing interest and encouragement of this research.

The author is indebted to Dr. Kazumitsu Hanai, Professor of Laboratory of Physics, Kyoto Prefectural University of Medicine, for many supports, valuable discussions, and useful suggestions during this work.

The author would like to express her acknowledges to Dr. Takashi Yamamoto, Professor of Laboratory of Behavioral Physiology, Graduate School of Human Sciences, Osaka University, for carrying out of sampling of rat CSF and helpful discussions.

The author is greatly grateful to Dr. Teruo Kawada, Associate Professor of the Laboratory for his useful advice and encouragement of this research.

The author is deeply grateful to Dr. Kazuo Inoue and Dr. Satoshi Tsuzuki, Assistant Professor of the Laboratory for their useful suggestion and advice.

The author wishes to thank Dr. Masahiro Imaizumi and Dr. Hanae Yamazaki, Research Associate of the Laboratory for their help, kind advice and encouragement throughout this study.

The author appreciates to Takayuki Kawai, Ayako Suzuki, Masami Takeda, Hiroya Kawasaki, Kyoko Kuroda, Shoko Sawano, Taro Toyoda and Yumie Kanamori, the member of the same research group for their experimental work and useful discussion.

The author also acknowledges all the members of Laboratory of Nutrition Chemistry, Kyoto University, for their helpful support and advise.

Finally, the author thanks her parents and sisters for their encouragement and support.